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Inventor: LEVAVA ROIZ ET AL.

METHODS OF AND COMPOSITIONS FOR INHIBITING THE PROLIFERATION OF MAMMALIAN CELLS

Enclosed are:

9 sheets of informal drawing(s).

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APPLICATION FOR PATENT

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METHODS OF AND COMPOSITIONS FOR INHIBITING
THE PROLIFERATION OF MAMMALIAN CELLS

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FIELD AND BACKGROUND OF THE INVENTION

The present invention relates to pharmaceutical compositions containing as an active ingredient a ribonuclease of the T2 family or a 25 polynucleotide encoding same, and further to methods of using such pharmaceutical compositions in inhibiting the proliferation of mammalian cells, such as cancer cells.

There is an ongoing interest, both within the medical community and among the general population, in the development of novel therapeutic 30 agents for the treatment of cell proliferative diseases such as cancer.

Agents that display anti-proliferative properties against mammalian cells can potentially be used as anti-cancer drugs. As such, these agents are widely sought for from both natural as well as synthetic sources.

- 5 RIBASES are ribonucleases (RNases) which display a biological activity which is distinct from their ability to degrade RNA. RIBASES and their structural homologous are known to effect a large number of cellular reactions (Rybak, M. *et al.*, . 1991, J. Biol. Chem. 266:21202-21207; Schein, C.H. 1997 Nature Biotechnol. 15:529-536). EDN and
- 10 ECP, two major proteins found in the secretory granules of cytotoxic eosinophiles (members of RNase A family) are thought to participate in the immune response. In self-incompatible plants stylar S-RNases (members of RNase T2 family), arrest pollen tube growth and thus prevent fertilization. RC-RNase, produced from Bullfrog oocytes, inhibits, *in*
- 15 *vitro*, the growth of tumor cells such as the P388, and L1210 leukemia cell lines and is effective for *in vivo* killing of sarcoma 180, Erlich, and Mep II ascites cells (Chang, C-F. *et al* 1988, J. Mol Biol 283:231-244). Some RNases display limited ribonuclease activity, an example of which

includes angiogenins that stimulate blood vessels formation (Fett, J.W. 1985, Biochemistry 24:5480-5486).

Living organisms use extracellular RNases for defense against pathogens and tumor cells. For example, ECP is secreted in response to 5 parasite attack (Newton, DL. 1992, J. Biol. Chem. 267:19572-19578) and displays antibacterial and antiviral activity. This activity is also displayed by Zinc- α 2-glycoprotein (Zn α 2gp), an RNase present in most human body fluids including blood, seminal plasma, breast milk, synovial fluid, saliva, urine and sweat (Lei G, et al., 1998, Arch Biochem Biophys. Jul 10 15;355(2):160-4).

The specific mechanism by which extracellular RNases function in cellular reactions is unknown.

The main barrier to the cytotoxic activity of some RNase is the cell membrane. ECP was found to form channels in both artificial and cellular 15 membranes. Presumably, ECP released from the granule membrane along with EDN (eosinophytic RNase, which is responsible for cerebellar Purkinje cell destruction) transfers EDN into the intercellular space. The entrance of the fungal toxin α -sarcin (a member of the RNase A family) into target cells depends upon viral infection which permeabilizes the

cellular membrane (Rybak, M. *et al.*, 1991, J. Biol. Chem. 266:21202-21207). It is also possible that RNases enter the cell via endocytosis. When the Golgi-disrupting drugs retinoic acid or monensin were used to artificially deliver BS-RNase into the cells, cytotoxicity increased 5 dramatically (Wu Y, *et al.*, 1995, J Biol Chem. 270(29):17476-81).

Cytotoxicity of RNases can be used for therapeutic purposes. Human RNase L is activated by interferon and inhibits viral growth. Expression of the gene for human RNase L together with that for a 2'5'-A synthetase in tobacco plants is sufficient to protect plants from cucumber 10 mosaic virus and to prevent replication of potato virus Y. Human immunodeficiency virus-1 (HIV-1) induces blockade in the RNase L antiviral pathways (Schein, C.H. 1997 Nature Biotechnol. 15:529-536.). RNases can be fused with specific membranal protein antibodies to create immunotoxins. For example, fusion of RNase A with antibodies to the 15 transferrin receptor or to the T cell antigen CD5 lead to inhibition of protein synthesis in tumor cells carrying a specific receptor for each of the above toxins (Rybak, M. *et al.*, 1991, J. Biol. Chem. 266:21202-21207; Newton DL, *et al.*, 1998, Biochemistry 14;37(15):5173-83). Since RNases

are less toxic to animals, they may have fewer undesirable side effect than the currently used immunotoxins.

The cytotoxicity of cytotoxic ribonucleases appears to be inversely related to the strength of the interaction between a ribonuclease inhibitor (RI) and the RNase. Ribonuclease inhibitor (RI) is a naturally occurring molecule found within vertebrate cells which serves to protect these cells from the potentially lethal effects of ribonucleases. The ribonuclease inhibitor is a 50 kDa cytosolic protein that binds to RNases with varying affinity. For example, RI binds to members of the bovine pancreatic ribonuclease A (RNase A) superfamily of ribonucleases with inhibition constants that span ten orders of magnitude, with K_i 's ranging from 10^{-6} to 10^{-16} M.

A-RNases

ONCONASE, like RNase A and BS-RNase, is a member of the RNase A superfamily. Members of the RNase A superfamily share about 30 % identity in amino acid sequences. The majority of non-conserved residues are located in surface loops, and appear to play a significant role in the dedicated biological activity of each RNase. ONCONASE was isolated from Northern Leopard frog (*Rana pipiens*) oocytes and early

embryos. It has anti-tumor effect on a variety of solid tumors, both *in situ* and *in vivo* (Mikulski S.M., *et al.*, 1990 J. Natl. Cancer 17;82(2):151-3). ONCONASE has also been found to specifically inhibit HIV-1 replication in infected H9 leukemia cells at non-cytotoxic concentration (Youle R.J., 5 *et al.*, 1994, Proc. Natl. Acad. Sci. 21;91(13):6012-6).

Although the RNase activity of ONCONASE is relatively low, it is accepted that the enzymatic and cytotoxic activities thereof are associated to some degree. It is believed that the tertiary structure of A-RNases differentiate between cytotoxic and non-cytotoxic types. For example, 10 differences between the tertiary structure of ONCONASE and RNase A are believed to be responsible for the increased cytotoxicity observed for ONCONASE. ONCONASE, unlike RNase A, contains a blocked N-terminal Glu1 residue (pyroglutamate) which is essential for both enzymatic and cytotoxic activities. This unique structure enables 15 ONCONASE to permeate into target cells (Boix E., *et al.*, 1996, J. Mol. Biol. 19:257(5):992-1007). In addition, in ONCONASE the Lys9 residue replaces the Gln11 residue of RNase A, which is believed to effect the structure of the active site. Furthermore, differences in the amino acid sequence of the primary structure between ONCONASE and RNase A

cause topological changes at the periphery of the active site which effect the specificity thereof (Mosimann S.C., *et al.*, 1992, Proteins 14(3):392-400).

The differences in toxicity between A-RNases are also attributed to
5 their ability to bind RI. Bovine seminal ribonuclease (BS-RNase) is 80 % identical in its amino acid sequence to RNase A, but unlike other members of the RNase A superfamily, BS-RNase exists in a dimeric form. It has been shown that the quaternary structure of BS-RNase prevents binding by RI, thereby allowing the enzyme to retain its ribonucleolytic activity in the
10 presence of RI (Kim *et al.*, 1995, J. Biol. Chem. 270 No. 52:31097-31102). ONCONASE, which shares a high degree of homology with RNase A, is resistant to binding by RI. The RI-ONCONASE complex has a K_d at least one hundred million times less than that of the RI-RNase A complex. The lower binding affinity of ONCONASE for RI prevents
15 effective inhibition of the ribonucleolytic activity and could explain why ONCONASE is cytotoxic at low concentrations while RNase A is not.

It is suggested that binding to cell surface receptor is the first step in ONCONASE cytotoxicity. Nothing is known about the nature of ONCONASE receptors on mammalian cell surfaces. ONCONASE may

bind to cell surface carbohydrates as in the case of ricin, or it may bind to receptors originally developed for physiologically imported molecules like polypeptide hormones (Wu Y, *et al.*, 1993, *J. Biol. Chem.* 15;268(14):10686-93). In mice, ONCONASE was eliminated from the 5 kidneys in a rate 50-100-fold slower than did RNase A. The slower elimination rate of ONCONASE is explained as a result of its higher ability to bind to the tubular cells and/or by its resistance to proteolytic degradation. The strong retention of ONCONASE in the kidneys might have clinical implications (Vasandani V.M., *et al.*, 1996, *Cancer Res.* 10 15;56(18):4180-6). ONCONASE may also bind to Purkinje cells EDN receptors (Mosimann S.C., *et al.*, 1996, *J. Mol. Biol.* 26; 260(4):540-52). The specificity of ONCONASE is also expressed in its tRNA preference. In rabbit reticulocyte lysate and in *Xenopus* oocytes it was discovered that ONCONASE inhibits protein synthesis via tRNA, rather than via rRNA or 15 mRNA degradation. In contrast, RNase A degrades mostly rRNA and mRNA (Lin J.J., *et al.*, 1994, *Biochem. Biophys. Res. Commun.* 14; 204(1):156-62).

Treatment of susceptible tissue cultures with ONCONASE results in the accumulation of cells arrested in G1 phase of the cell cycle, having

very low level of RNA contents (Mosimann S.C., *et al.*, 1992, Proteins 14(3):392-400). In glioma cells ONCONASE inhibited protein synthesis without a significant reduction in cell density, showing that ONCONASE is also cytotoxic to cells in addition to being cytostatic (Wu Y., *et al.*, 5 1993, J. Biol. Chem. 15;268(14):10686-93). ONCONASE, combined with chemotherapeutic agents, can overcome multidrug resistance. Treatment with vincristine and ONCONASE increased the mean survival time (MST) of mice carrying vincristine resistant tumors to 66 days, compared to 44 days in mice treated with vincristine alone (Schein, C.H., 1997, Nature 10 Biotechnol. 15:529-536). Furthermore, some chemotherapeutic agents may act synergistically with ONCONASE. In tumor cell lines of human pancreatic adenocarcinoma and human lung carcinoma treated with a combination of ONCONASE and tamoxifen (anti-estrogen), trifluoroperazine (Stelazine, calmodulin inhibitor) or lovastatin (3-15 hydroxyl-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitor) a stronger growth inhibition was observed than cells treated with ONCONASE alone (Mikulski S.M., *et al.*, 1990, Cell Tissue Kinet.;23(3):237-46). Thus, a possibility of developing combination therapy regimens with greater efficiency and/or lower toxicity is clear.

Bovine seminal RNase is a unique member of RNase A family, since it is the only RNase containing a dimer of RNase A-like subunits linked by two disulfide bridges. In addition, it maintains allosteric regulation by both substrate and reaction products. The regulation occurs

5 at the cyclic nucleotide hydrolysis phase. It has the ability to cleave both single- and double-stranded RNA. BS-RNase is highly cytotoxic. It displays anti-tumor effect *in vitro* on mouse leukemic cells, HeLa and human embryo lung cells, mouse neuroblastoma cells, and human fibroblasts and mouse plasmacytoma cell lines. When administrated *in*

10 *vivo* to rats bearing solid carcinomas (thyroid follicular carcinoma and its lung metastases), BS-RNase induced a drastic reduction in tumor weight, with no detectable toxic effects on the treated animals (Laccetti, P. *et al.*, 1992, Cancer Research 52:4582-4586). Artificially monomerized BS-RNase has higher ribonuclease activity but lower cytotoxicity than native

15 dimeric BS-RNase (D'Allessio G., *et al.*, 1991, TIBS:104-106). This, again, indicates the importance of molecular structure for the biological activity. It seems that like ONCONASE, BS-RNase binds to recognition site(s) on the surface of the target cells, prior to penetration into target cells.

In addition to being cytotoxic, BS-RNase is also immunorepressive.

BS-RNase can block the proliferation of activated T cells, and prolong the survival of skin grafts transplanted into allogenic mice. The immunorepressive activity of SB-RNase is explained by the need to protect sperm cells from the female immune system.

T2-RNases

In plants, self-compatibility is abundant and is effective in preventing self-fertilization. Pollen carrying a particular allele at the S locus, which controls self-incompatibility, is unable to fertilize plants carrying the same S-allele. In many self-incompatible plants, especially members of *Solanaceae* and *Rosaceae*, S-RNase, a member of the T2-RNase family is secreted by the female organs. S-RNase specifically recognize self-pollen and arrest its growth in the stigma or style before fertilization occurs (Clarke, A.E. and Newbigin, E., 1993, Ann. Rev. Genet. 27:257-279) it is believed that the arrest of pollen tube growth is a direct consequence of RNA degradation, however the mode of S-RNase entrance into the tube cell is still obscure.

Members of RNase T2 family were first identified in fungi (Egami, F. and Nakamura, K. 1969, Microbial ribonucleases. Springer-Verlag,

Berlin). Since, they were found in a wide variety of organisms, ranging from viruses to mammals. In particular, T2-RNases show much broader distribution than the extensively described RNase A family. However, the *in vivo* role of T2-RNases in mammalian cells is still not known.

5 In microorganisms, extracellular T2-RNases are generally accepted to contribute to the digestion of polyribonucleotides present in the growth medium, thereby giving rise to diffusible nutrients. They may also serve as defense agents (Egami, F. and Nakamura, K., 1969, Microbial ribonucleases. Springer-Verlag, Berlin).

10 In plants, T2-RNases play a role in the pollination process, by selectively limiting the elongation of pollen tubes racing towards the ovules (Roiz, L. and Shoseyov, O., 1995, Int. J. Plant Sci. 156:37-41, Roiz L. *et al.*, 1995, Physiol. Plant. 94:585-590). To date, the mechanism by which these RNases affect pollen tubes is unclear.

15 Thus, there exist few examples of cytotoxic ribonucleases which can be effectively used as cancer treatment agents. New cytotoxic ribonucleases with anti-proliferative activity in mammalian cells are needed to enhance the spectrum of therapeutic agents available for

treatment of human cancers to thereby open new horizons in the field of cancer treatment.

There is thus a widely recognized need and it would be highly advantageous to have a novel, cytotoxic, ribonuclease that has potential 5 usefulness in the treatment of human disease such as cancer.

SUMMARY OF THE INVENTION

According to one aspect of the present invention there is provided a 10 method of inhibiting the proliferation of a mammalian cell, the method comprising the step of administering to a treated mammal a pharmaceutical composition including, as an active ingredient, a ribonuclease of the T2 family, the composition further including a pharmaceutically acceptable carrier.

15 According to another aspect of the present invention there is provided a method of inhibiting the proliferation of a mammalian cell, the method comprising the step of administering to a treated mammal a pharmaceutical composition including, as an active ingredient, a polynucleotide including a first polynucleotide segment encoding a

ribonuclease of the T2 family, the composition further including a pharmaceutically acceptable carrier.

According to yet another aspect of the present invention there is provided a pharmaceutical composition comprising, as an active 5 ingredient, a ribonuclease of the T2 family, and a pharmaceutically acceptable carrier.

According to still another aspect of the present invention there is provided a pharmaceutical composition comprising, as an active ingredient, a polynucleotide sequence including a first polynucleotide 10 segment encoding a ribonuclease of the T2 family, and a pharmaceutically acceptable carrier.

According to an additional aspect of the present invention there is provided a novel use of a ribonuclease of the T2 family in the preparation of a medicament useful in inhibiting the proliferation of a mammalian cell, 15 the preparation comprising the step of mixing the ribonuclease of the T2 family with a pharmaceutically acceptable carrier.

According to yet an additional aspect of the present invention there is provided a novel use of a polynucleotide including a first polynucleotide segment encoding a ribonuclease of the T2 family in the preparation of a

medicament useful in inhibiting the proliferation of a mammalian cell, comprising the step of mixing the polynucleotide with a pharmaceutically acceptable carrier.

According to further features in preferred embodiments of the
5 invention described below, the ribonuclease of the T2 family substantially lacks ribonucleolytic activity.

According to still further features in the described preferred
embodiments the mammalian cell is a cancer cell.

According to still further features in the described preferred
10 embodiments the mammalian cell is a proliferating cell associated with a
proliferative disorder selected from the group consisting of papilloma,
blastoglioma, Kaposi's sarcoma, melanoma, lung cancer, ovarian cancer,
prostate cancer, squamous cell carcinoma, astrocytoma, head cancer, neck
cancer, bladder cancer, breast cancer, lung cancer, colorectal cancer,
15 thyroid cancer, pancreatic cancer, gastric cancer, hepatocellular
carcinoma, leukemia, lymphoma, Hodgkin's disease, Burkitt's disease,
arthritis, rheumatoid arthritis, diabetic retinopathy, angiogenesis,
restenosis, in-stent restenosis and vascular graft restenosis.

According to still further features in the described preferred embodiments the step of administering to the treated mammal the pharmaceutical composition is effected by an administration mode selected from the group consisting of oral administration, topical 5 administration, transmucosal administration, parenteral administration, rectal administration and by inhalation.

According to still further features in the described preferred embodiments the ribonuclease T2 family is RNase B1.

According to still further features in the described preferred 10 embodiments the ribonuclease of the T2 family is selected from the group consisting of RNase T2, RNase Rh, RNase M, RNase Trv, RNase Irp, RNase Le2, RNase Phyb, RNase LE, RNase MC, RNase CL1, RNase Bsp1, RNase RCL2, RNase Dm, RNase Oy and RNase Tp.

According to still further features in the described preferred 15 embodiments the pharmaceutically acceptable carrier includes a delivery vehicle capable of delivering the polynucleotide to the mammalian cell of the treated mammal.

According to still further features in the described preferred embodiments the delivery vehicle is selected from the group consisting of liposomes, micelles and virus.

According to still further features in the described preferred 5 embodiments the delivery vehicle is selected from the group consisting of an antibody and a ligand capable of specifically binding to the mammalian cell.

According to still further features in the described preferred embodiments the polynucleotide includes one or more additional 10 polynucleotide segments harboring transcription control sequences operatively linked to the first polynucleotide segment.

— According to still further features in the described preferred embodiments the polynucleotide is a eukaryotic expression vector.

The present invention successfully addresses the shortcomings of 15 the presently known configurations by characterizing a novel, cytotoxic, ribonuclease that has potential usefulness in the treatment of human disease such as cancer.

BRIEF DESCRIPTION OF THE DRAWINGS

The invention is herein described, by way of example only, with reference to the accompanying drawings, wherein:

FIG. 1 is a graph representation of the absorbance and RNase activity of *Aspergillus niger* RNase B1 isolated according to the teachings of Roiz, L. and Shoseyov, O., 1995, Int. J. Plant Sci. 156:37-41. Graph A represents fractions obtained via EMD-TMAE column chromatography of a crude filtrate, while graph B represents the fraction obtained from MONO-Q column chromatography of the active fractions resulted from the EMD-TMAE chromatography of the crude filtrate. The solid line represents absorbance at 280 nm and the dashed line represents RNase activity.

FIG. 2 is an SDS-PAGE zymogram illustrating the increase in RNase B1 protein concentration throughout the purification steps employed. Lane 1 represents the crude filtrate; lane 2 represents the eluate from the EMD-TMAE column; lane 3 represents the eluate from the MONO-Q column; lane 4 represent the eluate of lane 3 assayed *in situ* for RNase activity and stained with toluidine blue; lane 5 represents the

purified RNase following deglycosilation by PNGase F. Lanes 1-3 and 5 are stained with coomassie blue.

FIG. 3 is a graph illustrating the *in vitro* effect of different concentrations of B1 RNase on peach pollen germination (solid line with 5 black squares) and pollen tube length (dashed line with boxes).

FIGs. 4a and 4b illustrate the effect of RNase B1 on peach pollen tube growth in the stigma and the upper part of the style. Figure 4a represents the control flower, while Figure 4b is a flower treated with RNase B1 before pollination. Bar = 0.2 mm

10 FIGs. 5a and 5b illustrate the effect of RNase B1 on pollen tube growth in the stigma of a tangerine flower. Figure 5a represents a control flower which was exposed to open pollination for 48 hours. Figure 5b represents a flower which was treated with RNase B1 prior to pollination. Bar = 0.1 mm.

15 FIGs. 6a and 6b illustrate viability test conducted on nectarine seeds. Figure 6a represents a control seed produced by an untreated flower, while Figure 6b represents a seed produced by an RNase B1 treated flower. Bar = 0.3 mm.

FIG. 7 illustrates the effect of RNase B1, untreated, boiled or autoclaved, on lily cv. Osnat pollen tube length.

FIGs. 8a and 8b illustrate the effect of RNase B1 on lily pollen tubes growing *in vitro* and stained with IKI.

5 FIGs. 9a and 9b illustrate still shots captured from integrated video images showing organelle movement and localization in RNase B1 untreated (Figure 9a) and treated (Figure 9b) pollen tubes.

10 FIGs. 10a and 10b illustrate the effect of RNase B1 on actin filaments of a growing lily pollen tube. Figure 10a represents the control pollen tube whereas Figure 10b represents the RNase B1 treated pollen tube. Both pollen tubes were excised and stained with TRITC phalloidine for visualization following experimentation.

15 FIG. 11 is a Scatchard plot representing RNase B1 binding to actin.
A - actin concentration (μM), Rf - free RNase B1 concentration (μM), Rb - bound RNase B1 concentration (μM).

FIGs. 12a-c illustrate immunogold silver stained lily pollen tubes grown for 1 hour. Figure 12a represents a control, whereas Figures 12b and 12c are both RNase B1 treated pollen tubes. The pollen tube of Figure

12b was incubated with rabbit pre-immune serum, while the pollen tube of Figure 12c was incubated with anti-RNase B1 rabbit polyclonal antibody.

FIGs. 13a and 13b illustrate the effect of different concentrations of RNase B1 on the viability of HT29 colon cancer cells. Replicate samples 5 of cells were grown for 48 hours or for 72 hours at 37 °C, visualized using trypan blue differential staining and counted. Figure 13a represents the total numbers of cells whereas Figure 13b represents the percent of dead cells.

FIG. 14 illustrates the effect of RNase B1 on clonogenicity of HT29 10 cells. Replicate samples of cells were preincubated in growth medium in the absence or presence of 10^{-6} M RNase B1 for 48 hours, trypsinized, washed, resuspended in RNase B1-free growth medium in serial dilutions, and plated into 96-well microtiter plates to colonize for 14 days. Colonies were counted following fixation and staining with methylene blue.

15 FIG. 15 illustrates the effect of exposure period to RNase B1 on the clonogenicity of HT29 cells. Replicate samples of cells were preincubated in growth medium containing 10^{-6} M RNase B1 for 48 hours and then let to colonize in growth medium containing the same concentration of RNase B1, or in RNase B1-free medium. Colonization was done in 96-well

microtiter plates for 7 days. Each treatment contained different initial numbers of cells per well. The colonies were counted following fixation and visualization in methylene blue. Cells preincubated and colonized in RNase B1-free growth medium served as a control.

5 FIGs. 16a-c illustrate the effect of RNase B1 on the colonization ability of HT29 cells. Control cells (Figure 16a) were preincubated 48 hours in RNase B1-free growth medium and then trypsinized and incubated in the same growth medium in 96-microtiter plates for colonization. Figure 16b represents cells that were preincubated for 48
10 hours in growth medium containing 10^{-6} M RNase B1 and then allowed to colonize in RNase B1-free growth medium. Figure 16c represents cells that were preincubated and then colonized in growth medium containing 10^{-6} M RNase B1. The cell colonies were visualized using methylene blue staining.

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DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention is of pharmaceutical compositions which include a ribonuclease of the T2 family or a polynucleotide encoding same and methods of using these pharmaceutical compositions to inhibit the proliferation of mammalian cells. Specifically, the present invention can be used to treat a mammal suffering from a proliferative disease or disorder, such as, but not limited to, cancer, by delivering or targeting the pharmaceutical compositions of the present invention to the proliferative cells associated with the disease or disorder.

10 The use of ribonuclease with cytotoxic activity for inhibiting the proliferation of tumor cells is not new and has been demonstrated previously in the art. A ribonuclease of the A family which is commercially referred to as ONCONASE has been shown to inhibit cell proliferation in tumorous tissue in clinical trials. Several other RNases of 15 the RNase A superfamily have also been demonstrated to have cytotoxic activity in addition to their ribonucleolytic activity.

Although the cytotoxicity of some cytotoxic ribonucleases is dependent to some extent on their ribonucleolytic activity, the level of ribonucleolytic activity does not always correlate to the level of

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cytotoxicity observed for these ribonucleases. Furthermore, there exist several examples of ribonucleases which do not display cytotoxic activity altogether, yet function as ribonucleases. The most known example is RNase A. In other cases, the reaction rate is scarified for more specific binding or improved function in some other capacity. For example, angigenin's active site is blocked by side chains that are not present in RNase A, rendering it 10,000-fold less active on general substrates, but more specific in cleaving ribosomal RNA. BS-RNase is a faster nuclease when it is monomeric. However, its cytotoxicity is higher and inhibition by ribonuclease inhibitor considerably reduced in the dimer form: presumably the dimeric form allows better passage through cell membranes. Glycosilated RNase B is less active than RNase A on most substrates, while a frequently observed deamidation in BS-RNase, (asparagine 67 to isoaspartate) reduces the activity of RNase A mutants by cleaving a whole chain of H-bond structures in the protein. (reviewed in Shein, C.H. 1997. Nature Biotechnol 15: 529-536).

Ribonucleases of the T2 are characterized by their unique molecular features. A comparison between RNase members of the A and

of the T2 families is summarized below in Table 1 (Location of amino acids are after RNase A and RNase T2 in families A and T2, respectively).

TABLE 1

5

Feature	RNase A	RNase T2
Molecular mass	11-14 kDa (with the exception of BS-RNase)	24-36 kDa.
Optimal temperature for RNase activity:	37°C	50-60°C
Optimal pH for RNase activity:	6.5-8	3.5-5
	Not glycosilated	12-25% of the total molecular mass
Base specificity:	Pyrimidine base-specific.	Non specific with adenylic acid preferential.
Disulfide bonds:	Four: Common: Cys28-84, Cys40-96, Cys58-110. In pancreatic RNases the fourth S-S bond is located between Cys65-72, forming a loop containing Glu69 and Asn71, which are part the nucleotide-binding site. In ONCONASE and bullfrog lectin Cys87-Cys104 form a COOH-terminal loop, which is located near the active site. Angiogenins have only 3 disulfide bonds.	Five: Cys3-20, Cys10-53, Cys19-120, Cys63-112 and Cys182-213.

Mechanism of RNase activity:	<u>Active site</u> <u>Two steps in RNA cleavage</u> (i) His12 acts as a general base and removes a proton from the 2'-hydroxyl group of the RNA. His119 acts as a general acid, donating a proton from the 5' O of the leaving nucleotide. (ii) The resultant 2'3'-cyclic nucleotides are hydrolyzed, with the roles of His12 and His119 reversed. Lys41 stabilizes the pentavalent transition state.	<u>Active site</u> RNA catalysis is similar to RNase A. His46 and His109 function as general acid and base catalysts. Glu105 and Lys108 might play a role in polarizing the P=O bond of the substrate or in stabilizing the pentacovalent transition state.
	<u>Substrate binding sites:</u> Gln11 and Phe120 form hydrogen bonds with the substrate. In ONCONASE and bullfrog lectine Glu11 forms H-bond with the phosphate of the substrate. Gln96, Asn71, Glu111, of which Asn71 is the most conserved, might catalyze RNA cleavage.	<u>Substrate binding sites:</u> His104 (In plants it is Tyr or Asp) might act as the phosphate receptor of the substrate. There are two recognition sites: The major (B1) site contains Tyr57, Trp49 and Asp51. Asp51 is responsible for the adenine base recognition. A minor (B2) site contains Phe101, Gln95, Asn94, Ser93, Pro92 and Gln32.

The ribonucleases of the T2 family have been identified in numerous microorganisms as well as plants in which they play an active role in the pollination process, by selectively limiting the elongation of pollen tubes racing towards the ovules.

5 As uncovered by the inventors of the present invention and as further detailed hereinbelow in Example 2, RNase B1, a T2 ribonuclease,

specifically binds to actin in elongating pollen tubes to thereby inhibit the elongation of pollen tubes.

In cells, actin forms filaments which are essential cytoskeletal components active in both maintaining cellular structure and in supporting 5 intracellular transport of organelles. As a result, actin filaments participate in many cellular processes throughout the life cycle of normal and abnormal cells. Numerous studies have shown that actin also participates in various cellular processes controlling generation and proliferation of cancer cells (Jordan, M.A. & Wilson, L. 1998. *Curr. Opin. Cell Biol.* 10:123-130; Jammy, P.A. & Chaponnier,C. 1995. *Curr. Opin. Cell Biol.* 7:111-117; Sigmund, S.H. 1996. *Curr. Opin. Cell Biol.* 8:66-73; Tapon, N. *et al.* 1997. *Curr. Opin. Cell Biol.* 9:86-92). Thus, for example, actin 15 filaments participates in abnormal cell proliferation (Assoian, R.K. & Zhu, X. 1997. *Curr. Opin. Cell Biol.* 9:93-98). Malignant cells were found more sensitive to cytochalasin B than normal cells were (Hemstreet G.P. *et al.* 1996. *J. Cell Biochem.* 25S:197-204).

Since actin is a highly conserved protein maintaining a high level of homology between evolutionary distant organisms it was hypothesized that the actin binding activity of RNase B1, which inhibits pollen tube

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elongation can be utilized to specifically bind actin in mammalian cells to therefor inhibit the proliferation thereof.

Thus, while reducing the present invention to practice and as further described in Example 3 hereinbelow, the effect of RNase B1 on 5 proliferating mammalian cancer cells was further investigated. As clearly demonstrated therein, RNase B1 substantially decreases proliferation in colon cancer cell cultures.

Before explaining at least one embodiment of the invention in detail, it is to be understood that the invention is not limited in its 10 application to the details of construction and the arrangement of the components set forth in the following description or illustrated in the drawings. The invention is capable of other embodiments or of being practiced or carried out in various ways. Also, it is to be understood that the phraseology and terminology employed herein is for the purpose of 15 description and should not be regarded as limiting.

One or more ribonucleases of the T2 family are collectively referred to herein as T2-RNase. Similarly, one or more polynucleotides encoding one or more ribonucleases of the T2 family are collectively referred to herein as polynucleotide encoding T2-RNase (or same).

Thus, according to one aspect of the present invention there is provided a method of inhibiting the proliferation of a mammalian cell. The method according to this aspect of the present invention is effected by administering to a treated mammal a pharmaceutical composition which

5 includes, as an active ingredient, a polynucleotide encoding a T2-RNase, which is translatable *in vivo* into anti-proliferative T2-RNase. As further detailed herein under, the pharmaceutical composition further includes a pharmaceutically acceptable carrier.

According to one preferred embodiment of the present invention

10 the polynucleotide according to this and other aspects of the present invention encodes a native T2-RNase protein, which term is used herein to describe a T2-RNase having both anti-proliferative and ribonucleolytic activities.

As is further detailed in Example 2 below, it has been shown by the

15 inventors of the present invention that the anti-proliferative activity of RNase B1 is not dependent on its ribonucleolytic activity, as boiled or autoclaved RNase B1, which has little or substantially no ribonucleolytic activity retained substantially all of its anti-proliferative activity.

Thus, according to an embodiment of the present invention, a polynucleotide encoding a silent or repressed T2-RNase mutant, having no or little ribonucleolytic activity is utilized as the active ingredient in the pharmaceutical composition of the present invention, to be translated *in vivo* into an antiproliferative protein which is free of ribonucleolytic activity.

As such, the term "polynucleotide" when used herein in context of T2-RNases in general, or in context of a specific T2-RNase, also refers to any polynucleotide sequence which encodes a T2-RNase active in inhibiting mammalian cell proliferation but which is devoid of, or 10 repressed in, ribonucleolytic activity. Such polynucleotides can be obtained using known molecular biology techniques, such as random mutagenesis, site-directed mutagenesis and enhanced evolution techniques. Site directed mutagenesis can be readily employed because 15 the amino acid residues essential for the ribonucleolytic activity of T2-RNases have been recognized (see Kusano et al., 1998. Biosci. Biothechnol. Biochem. 62:87-94, which is incorporated herein by reference).

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According to another aspect of the present invention there is provided another method of inhibiting the proliferation of a mammalian cell. The method according to this aspect of the present invention is effected by administering to a treated mammal a pharmaceutical composition which includes, as an active ingredient, a T2-RNase and which further includes a pharmaceutically acceptable carrier. The T2-RNase can be derived from a native source, as is further exemplified in Example 1 that follows, or alternatively, it can be produced as a recombinant protein using any one of the above described polynucleotides.

10 Thus, it will be appreciated that a T2-RNase protein according to this and other aspects of the present invention can be utilized both in a native ribonucleolytic active form, or alternatively, in a silent, or repressed ribonucleolytic mutant form, having no or little ribonucleolytic activity, yet which retains its anti-proliferative activity.

15 Thus, a T2-RNase can be used following inactivation or denaturation which abolishes its ribonucleolytic activity, yet retains its anti-proliferative activity. For example, a T2-RNase can be boiled or autoclaved prior to the inclusion in a pharmaceutical composition according to the present invention. These forms of a T2-RNase can be

used provided they exhibit anti-proliferative activity. As such, the term "T2-RNases" is meant to encompass all the anti-proliferative forms of the protein.

It will be appreciated that utilizing a T2-RNase, either directly or
5 expressed from a polynucleotide, which displays anti-proliferative activity
and yet is devoid of, or repressed in, ribonucleolytic activity is particularly
advantageous since ribonucleolytic activity can produce undesired side
effects in a treated mammal.

As used herein the phrase "pharmaceutical composition" refers to a
10 preparation including an active ingredient combined with other
components, generally referred to herein as "pharmaceutically acceptable
carriers". The purpose of a pharmaceutical composition is to facilitate
administration of the active ingredient contained therein to a cell or tissue
of a mammal.

15 Some pharmaceutical compositions of the present invention may be
manufactured by processes well known in the art, e.g., by means of
conventional mixing, dissolving, granulating, dragee-making, levigating,
emulsifying, encapsulating, entrapping or lyophilizing processes.

Herein, the term "pharmaceutically acceptable carrier" refers to a carrier or a diluent that does not abrogate, and preferably enhance, the anti-proliferative activity and properties of the administered active ingredient.

5 According to one preferred embodiment of the present invention the mammalian cell is a cancer or a tumor cell.

As used herein the terms "cancer" or "tumor" are clinically descriptive terms which encompass a myriad of diseases characterized by cells that exhibit abnormal cellular proliferation. Such diseases can
10 include, but are not limited to, papilloma, blastoglioma, Kaposi's sarcoma, melanoma, lung cancer, ovarian cancer, prostate cancer, squamous cell carcinoma, astrocytoma, head cancer, neck cancer, bladder cancer, breast cancer, lung cancer, colorectal cancer, thyroid cancer, pancreatic cancer, gastric cancer, hepatocellular carcinoma, leukemia, lymphoma, Hodgkin's
15 disease and Burkitt's disease.

The term "tumor", when applied to tissue, generally refers to any abnormal tissue growth, characterized in excessive and abnormal cellular proliferation. A tumor may be "benign" and unable to spread from its original focus, or "malignant" or "metastatic" and capable of spreading

beyond its anatomical site to other areas throughout the host body. The term "cancer" is an older term which is generally used to describe a malignant tumor or the disease state arising therefrom. Alternatively, the art refers to an abnormal growth as a neoplasm, and to a malignant 5 abnormal growth as a malignant neoplasm.

According to another preferred embodiment of the present invention the mammalian cell is a proliferating cell associated with a proliferative disorder such as, but not limited to, arthritis, rheumatoid arthritis, diabetic retinopathy, angiogenesis, restenosis, in-stent restenosis 10 and vascular graft restenosis.

According to a preferred embodiment of the present invention the ribonuclease of the T2 family is ribonuclease B1.

Alternatively and according to another preferred embodiment of the present invention the T2-RNase is RNase T2, RNase Rh, RNase M, RNase 15 Trv, RNase Irp, RNase Le2, RNase Phyb, RNase LE, RNase MC, RNase CL1, RNase Bsp1, RNase RCL2, RNase Dm, RNase Oy or RNase Tp. It will be appreciated that combinations of any of the ribonucleases of the T2 family can also be used as the active ingredient of the pharmaceutical composition of the present invention. The listed RNases have been

characterized and the genes encoding same cloned. Table 2 below summarizes the presently known T2-RNases and their genes.

TABLE 2

TABLE 2

Source	Name (Prot)	Name (Gene)	Reference	Accession No.
<i>Bacteria</i>	<i>Aeromonas hydrophila</i>		Locus RNI	Favre,D. et al. 1993. J. Bacteriol. 175:3710-3722.
	<i>Haemophilus influenzae</i>	Rnase HI0526	Locus RN26	Fleischmann, R.D., et al. 1995. Science 269:496-512.
	<i>Escherichia coli</i>	Rnase I	Locus RNI	Meador,J. III. & Kennell,D. 1990. Gene 95:1-7. Oshima, T., et al. 1996. DNA Res. 3:137-155. Henikoff, S. & Henikoff, J.G. 1994. Genomics 19:97-107.
	<i>Aspergillus oryzae</i>	Rnase T2	rnt B	Kawata Y. et al. 1988. Eur J. Biochem 176(3):683-97. Kawata Y. et al. 1990. Eur J. Biochem 187:255-62. Ozeki K., et al. 1991. Curr Genet. 19:367-73.
<i>Fungi</i>	<i>Rhizopus niveus</i>	Rnase Rh		Horiuchi,H. et al. 1988. J. Biochem. 103:408-418. Kurihara,H. et al. 1992. FEBS Lett. 306:189-192. Kurihara,H. et al. 1996. J. Mol. Biol. 255:310-320. Ohgi, K. et al. 1991. J. Biochem. 109:776-785.
	<i>Trichoderma viride</i>	Rnase Trv		Inada,Y. et al. 1991. J. Biochem. 110 (6), 896-904.
	<i>Lentinula edodes</i> (shiitake mushroom)	Rnase Irp		Kobayashi, H. et al. 1992. Biosci. Biotechnol. Biochem. 56:2003-2010.
	<i>L. edodes</i>	Rnase Le2		Kobayashi,H. et al. 1992. Biosci. Biotechnol. Biochem. 56:2003-2010. Shimada,H. et al. 1991. Agric. Biol. Chem. 55:1167-1169.
	<i>Irpex lacteus</i>	Rnase Irp1		Watanabe,H., et al. 1995. Biosci. Biotechnol. Biochem. 59:2097-2103.
	<i>Physarum polycephalum</i>	Rnase Phyb		Inokuchi,N. et al. 1993. J. Biochem. 113:425-432.

<i>Plants</i>	<i>Arabidopsis thaliana</i>	RNS2	Locus RNS2	Green,P.J., et al. Proc. Natl. Acad. Sci. U.S.A. 90:5118-5122.	P42814
	<i>A. thaliana</i>	Rnase 3	Locus RNS3	Bariola,P.A., et al. 1994. Plant J. 6:673-685.	P42815
	<i>A. thaliana</i>	Rnase 1	Locus RNS1	Bariola,P.A., et al. 1994. Plant J. 6:673-685.	P42813
	<i>Lycopersicon esculentum</i> (cultured tomato)	Rnase LE	RNALE	Kock,M. et al. 1995. Plant Mol. Biol. 27:477-485. Jost,W. et al. 1991. Eur. J. Biochem. 198:1-6.	P80022
	<i>L. esculentum</i>	Rnase LX	RNLX	Kock,M., et al. 1995. Plant Mol. Biol. 27:477-485. Loffler,A., et al. 1993 Eur. J. Biochem. 214:627-633.	P80196
	<i>Nicotiana alata</i> (tobacco)	S-RNase	S	Anderson ,M.A., et al. 1986. Nature 321:38-44. Matton,D.P. et al. 1995. Plant Mol. Biol. 28:847-858. McClure ,B.A. et al. 1989. Nature 342:95-97.	P04002
	<i>Malus domestica</i> (apple tree)	S-RNases	S	Sassa,H., et al. 1996. Mol. Gen. Genet. 250:547-557.	
	<i>Pyrus pyrifolia</i> (Japanese pear)	S-RNases	S	Norioka,N., et al. 1996. J. Biochem. 120 ,335-345.	
	<i>Momordica charantia</i> (bitter gourd)	RNase MC	Locus RNMC	Blaxter,M.L., et al. 1996. Mol. Biochem. Parasitol. 77:77-93. Ide,H. et al. 1991. FEBS Lett. 284:161-164. Ide,H. et al. 1991. FEBS Lett. 289:126.	P23540

<i>Animals</i>	<i>Gallus gallus</i> (chicken)	RNase CL1		Uchida,T. et al. 1996. Biosci. Biotechnol. Biochem. 60:1982-1988.	JC5126
	<i>Rana catesbeiana</i> (bull frog)	RNase RCL2		Yagi,H. et al. 1995. Biol. Pharm. Bull. 18:219-222. Liao,Y.D. et al. 1998. J. Biol. Chem. 273. In press.	PC2347
	<i>Drosophila melanogaster</i>	RNase DM	DmRNase	Lankenau,D.H. et al. 1990. Chromosoma 99:111-117. Hime,G., et al. 1995. Gene 158:203-207.	X15066
	<i>Crassostera gigas</i> (pacific oyster.)	RNase Oy	Locus JX0295	Watanabe,H. et al. 1993. J. Biochem. 114:800-807.	JX029
	<i>Todarodes pacificus</i> (Japanese flying squid)	RNase Tp		Kusano, A. et al. 1998. Biosci. Biotechnol. Biochem. 62:87-94.	PMID 9501521
	<i>Homo sapiens</i>	RNase 6 precursor	RNASE6PL	Trubia, M. et al. 1997. Genomics 42:342-344.	00372 1

According to the present invention the pharmaceutical composition is administered to a mammal in order to inhibit the proliferation of cells such as a cancer cells.

To effect such administration the pharmaceutical composition

- 5 includes a suitable pharmaceutical carrier and an effective amount of a T2-
RNase or a polynucleotide encoding same, and is administered, for
example, topically, intraocularly, parenterally, orally, intranasally,
intravenously, intramuscularly, subcutaneously or by any other effective
means via methods well known in the art.

10 For intravenously, intramuscularly or subcutaneously injection, a
T2-RNase or a polynucleotide encoding same may be formulated in
aqueous solutions, preferably in physiologically compatible buffers such
as Hank's solution, Ringer's solution, or physiological saline buffer. For
example, a physiologically appropriate solution containing an effective
concentration of a T2-RNase or a polynucleotide encoding same can be
administered systemically into the blood circulation to treat a cancer or
tumor which cannot be directly reached or anatomically isolated. A
physiologically appropriate solution containing an effective concentration
of a T2-RNase or a polynucleotide encoding same may be directly injected

into a target cancer or tumor tissue by a needle in amounts effective to treat the tumor cells of the target tissue.

For transmucosal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are
generally known in the art.

For oral administration, the pharmaceutical composition of the present invention can be formulated readily by combining a T2-RNase or a polynucleotide encoding same with pharmaceutically acceptable carriers well known in the art. Such carriers enable a T2-RNase or a
polynucleotide encoding same to be formulated as tablets, pills, dragees,
capsules, liquids, gels, syrups, slurries, suspensions, and the like, for oral
ingestion by a patient. Pharmacological preparations for oral use can be
made using a solid excipient, optionally grinding the resulting mixture,
and processing the mixture of granules, after adding suitable auxiliaries if
desired, to obtain tablets or dragee cores. Suitable excipients are, in
particular, fillers such as sugars, including lactose, sucrose, mannitol, or
sorbitol; cellulose preparations such as, for example, maize starch, wheat
starch, rice starch, potato starch, gelatin, gum tragacanth, methyl cellulose,
hydroxypropylmethyl-cellulose, sodium carbomethylcellulose; and/or

physiologically acceptable polymers such as polyvinylpyrrolidone (PVP).

If desired, disintegrating agents may be added, such as cross-linked polyvinyl pyrrolidone, agar, or alginic acid or a salt thereof such as sodium alginate.

- 5 Dragee cores are provided with suitable coatings. For this purpose, concentrated sugar solutions may be used which may optionally contain gum arabic, talc, polyvinyl pyrrolidone, carbopol gel, polyethylene glycol, titanium dioxide, lacquer solutions and suitable organic solvents or solvent mixtures. Dyestuffs or pigments may be added to the tablets or dragee
10 coatings for identification or to characterize different combinations of active ingredient doses.

- Additional pharmaceutical compositions, which can be used orally, include push-fit capsules made of gelatin as well as soft, sealed capsules made of gelatin and a plasticizer, such as glycerol or sorbitol. The push-fit
15 capsules may contain a T2-RNase or a polynucleotide encoding same in admixture with filler such as lactose, binders such as starches, lubricants such as talc or magnesium stearate and, optionally, stabilizers. In soft capsules, a T2-RNase or a polynucleotide encoding same may be dissolved or suspended in suitable liquids, such as fatty oils, liquid

paraffin, or liquid polyethylene glycols. In addition, stabilizers may be added. All formulations for oral administration should be in dosages suitable for the chosen route of administration.

Oral delivery of the pharmaceutical composition of the present invention may not be successful due to the pH and enzyme degradation present in the gastrointestinal tract. Thus, such pharmaceutical compositions must be formulated to avoid undesirable circumstances. For example, enteric coating can be applied to oral solid formulation. Substances with acidic-resistant properties such as cellulose acetate phtalate (CAP), hydroxypropyl methycellulose phtalate (HPMCP) and acrylic resins are most commonly used for coating tablets or granules for micro encapsulation. Preferably wet granulation is used to prepare the enteric-coated granules to avoid reactions between the active ingredient and the coating (Lin, S.Y. and Kawashima, Y. 1987, Pharmaceutical Res. 4:70-74). A solvent evaporation method can also be used. The solvent evaporation method was used to encapsulate insulin administered to diabetic rats to maintain blood glucose concentration (Lin, S.Y. et al., 1986, Biomater, Medicine Device, Artificial organ 13:187-201 and Lin, S.Y. et al., 1988, Biochemical Artificial Cells Artificial Organ 16:815-

828). It was also used to encapsulate biological materials of high molecular weight such as vial antigen and concanavalin A (Maharaj, I. *Et al.* 1984, J. Phamac. Sci. 73:39-42).

For buccal administration, the pharmaceutical composition of the 5 present invention may take the form of tablets or lozenges formulated in conventional manner.

For rectal administration propositories can be used as is well known in the art.

For administration by inhalation, a T2-RNase or a polynucleotide 10 encoding same for use according to the present invention is conveniently delivered in the form of an aerosol spray presentation from a pressurized pack or a nebulizer with the use of a suitable propellant, e.g., dichlorodifluoromethane, trichlorofluoromethane, dichloro-tetrafluoroethane or carbon dioxide. In the case of a pressurized aerosol, 15 the dosage unit may be determined by providing a valve to deliver a metered amount. Capsules and cartridges of, e.g., gelatin for use in an inhaler or insufflator may be formulated containing a powder mix of a T2-RNase or a polynucleotide encoding same and a suitable powder base such as lactose or starch.

The pharmaceutical composition of the present invention may also be formulated for parenteral administration, e.g., by bolus injection or continuos infusion. A composition for injection may be presented in unit dosage form, e.g., in ampoules or in multidose containers with optionally, 5 an added preservative. The compositions may be suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents.

Pharmaceutical compositions for parenteral administration include aqueous solutions of the active preparation in water-soluble form. 10 Additionally, suspensions of a T2-RNase or a polynucleotide encoding same may be prepared as appropriate oily injection suspensions. Suitable lipophilic solvents or vehicles include fatty oils such as sesame oil, or synthetic fatty acids esters such as ethyl oleate, triglycerides or liposomes. Aqueous injection suspensions may contain substances, which increase the 15 viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol or dextran. Optionally, the suspension may also contain suitable stabilizers or agents which increase the solubility of a T2-RNase or a polynucleotide encoding same to allow for the preparation of highly concentrated solutions.

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Alternatively, a T2-RNase or a polynucleotide encoding same may be in powder form for constitution with a suitable vehicle, e.g., sterile, pyrogen-free water, before use.

- The pharmaceutical composition of the present invention may also
- 5 be formulated in rectal compositions such as suppositories or retention enemas, using, e.g., conventional suppository bases such as cocoa butter or other glycerides.

In addition, a cancer or tumor present in a body cavity such as in the eye, gastrointestinal tract, genitourinary tract (e.g., the urinary bladder), pulmonary and bronchial system and the like can receive a physiologically appropriate composition (e.g., a solution such as a saline or phosphate buffer, a suspension, or an emulsion, which is sterile) containing an effective concentration of a T2-RNase or a polynucleotide encoding same via direct injection with a needle or via a catheter or other delivery tube placed into the cancer or tumor afflicted hollow organ. Any effective imaging device such as X-ray, sonogram, or fiber optic visualization system may be used to locate the target tissue and guide the needle or catheter tube.

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The pharmaceutical composition of the present invention can also be delivered by osmotic micro pumps. The osmotic micro pumps are implanted into one of the body cavities and the drug is constantly released onto the tissue. This method is particularly advantageous when an 5 immune response to the pharmaceutical composition is experienced. This method has been employed for ONCONASE (Vasandani V.M., *et al.*, 1996, *Cancer Res.* 56(18):4180-6).

Alternatively and according to another preferred embodiment of the present invention, the pharmaceutically acceptable carrier includes a 10 delivery vehicle capable of delivering a T2-RNase or a polynucleotide encoding same to the mammalian cell of the treated mammal.

Numerous delivery vehicles and methods are known in the art for targeting proteins or nucleic acids into or onto tumor or cancer cells. For example, liposomes are artificial membrane vesicles that are available to 15 deliver proteins or nucleic acids into target cells (Newton, A.C. and Huestis, W.H., *Biochemistry*, 1988, 27:4655-4659; Tanswell, A.K. *et al.*, 1990, *Biochmica et Biophysica Acta*, 1044:269-274; and Ceccoli, J. *et al.*, *Journal of Investigative Dermatology*, 1989, 93:190-194). Thus, a T2-RNase or a polynucleotide encoding same can be encapsulated at high

efficiency with liposome vesicles and delivered into mammalian cells. In addition, the T2-RNase protein or nucleic acid can also be delivered to target tumor or cancer cells via micelles as described in, for example, U.S. Pat No. 5,925,628 to Lee, which is incorporated herein by reference.

5 Liposome or micelle encapsulated T2-RNase or a polynucleotide encoding same may be administered topically, intraocularly, parenterally, intranasally, intratracheally, intrabronchially, intramuscularly, subcutaneously or by any other effective means at a dose efficacious to treat the abnormally proliferating cells of the target tissue. The liposomes
10 may be administered in any physiologically appropriate composition containing an effective concentration of encapsulated T2-RNase or a polynucleotide encoding same.

Alternatively and according to another preferred embodiment of the present invention the delivery vehicle can be, but it is not limited to, an
15 antibody or a ligand capable of binding a specific cell surface receptor or marker. An antibody or ligand can be directly linked to a T2-RNase protein or nucleic acid via a suitable linker, or alternatively such an antibody or ligand can be provided on the surface of a liposome encapsulating a T2-RNase or a polynucleotide encoding same.

For example, a T2-RNase or a polynucleotide encoding same can be fused with specific membranal protein antibodies or ligands for targeting to specific tissues or cells as previously described in the art. It will be appreciated in this respect that fusion of RNase A of the 5 ribonuclease A superfamily with antibodies to the transferrin receptor or to the T cell antigen CD5 lead to inhibition of protein synthesis in tumor cells carrying a specific receptor for each of the above toxins (Rybak, M. *et al.*, 1991, *J. Biol. Chem.* 266:21202-21207 and Newton DL, *et al.*, 1997, *Protein Eng.* 10(4):463-70).

10 Thus, according to another aspect of the present invention a T2-RNase or a polynucleotide encoding same is used in the preparation of a medicament useful in inhibiting the proliferation of mammalian cells.

A polypeptide representing the amino acid sequence of a T2-RNase as defined herein can be produced by any one of several methods well known in the art. For example the polypeptide can be produced 15 synthetically by standard peptide synthesis techniques, for example using either standard 9-fluorenylmethoxycarbonyl (F-Moc) chemistry (see, for example, Atherton, E. and Sheppard, R. C. 1985, *J. Chem. Soc. Chem. Comm.* 165) or standard butyloxycarbonate (T-Boc) chemistry, although it

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is noted that, more recently, the fluorenylmethoxycarbonyl (Fmoc)/tert-butyl system, developed by Sheppard has found increasingly wide application (Sheppard, R. C. 1986 Science Tools, The LKB Journal 33, 9).

Alternatively, a T2-RNase protein can also be isolated and purified
5 by methods well known in the art from organisms known to express this protein. Such organisms include, for example, *Aeromonas hydrophila*, *Haemophilus influenzae*, *Escherichia coli*, *Aspergillus oryzae*, *Aspergillus phoenicis*, *Rhisopus niveus*, *Trichoderma viride*, *Lentinula edodes*, *Irpeus lacteus*; *Physarum polycephalum*, *Arabidopsis thaliana*, *Lycopersicon esculentum*, *Nicotiana alata*, *Malus domestica*, *Pyrus pyrifolia*, *Momordica charantia*, *Gallus gallus*, *Rana catesbeiana*, *Drosophyla melanogaster*, *Crassostera gigas*, *Todarodes pacificus* and *Homo sapiens*.
10 It is, however, anticipated that other organisms yet not known to produce T2-RNase, once uncovered as such, could also be used as a source for T2-RNase according to the present invention.
15

Alternatively and preferably a T2-RNase protein can be recombinantly produced by expressing a polynucleotide encoding same, using an appropriate expression vector system. Preferably, an expression system is selected which provides suitable post translational modifications.

Suitable expression vector systems include, but are not limited to, mammalian cells infected with a virus (e.g., adenovirus, retrovirus, herpes simplex virus, avipox virus); insect cells infected with a virus (e.g., baculovirus); genetically modified plants or plant cells transformed with a 5 plasmid, a plant virus or an *Agrobacterium*; transformed microorganisms such as yeasts containing yeast vectors, or bacteria transformed with bacteriophage DNA, plasmid DNA, or cosmid DNA. The expression controlling elements of vectors vary in their strengths and specifications depending on the host-vector system utilized, any one of a number of 10 suitable transcription and translation elements may be used. A recombinantly produced T2-RNase may be purified from host cells by affinity chromatography, electrophoresis, high-performance liquid chromatography (HPLC), immunoprecipitation, sedimentation or any other method known to the art.

15 A purified T2-RNase can be used to prepare a medicament according to the present invention by means of conventional mixing, dissolving, granulating, dragee-making, levigating, emulsifying, encapsulating, entrapping or lyophilizing processes with the addition of the appropriate pharmaceutically acceptable carriers and/or excipients or

alternatively it can be linked to appropriate delivery vehicles as described hereinabove.

As has already been mentioned hereinabove, according to an aspect of the present invention, the active ingredient of the pharmaceutical
5 composition effective in inhibiting the proliferation of a mammalian cell is a polynucleotide encoding a T2-RNase.

According to this aspect of the present invention the polynucleotide is introduced into the mammalian cell along with a pharmaceutically acceptable carrier, which introduction results in a genetic modification of
10 this cell, enabling the expression of a T2-RNase therein.

As used herein in the specification and in the claims section below, the term "genetic modification" refers to a process of inserting nucleic acids into cells. The insertion may, for example, be effected by viral infection, injection, transfection, or any other means effective in
15 introducing nucleic acids into cells some of which are further detailed hereinbelow. Following the genetic modification the nucleic acid is either integrated in all or part, to the cell's genome (DNA), or remains external to the cell's genome, thereby providing stably modified or transiently modified cells.

As such, the pharmaceutical composition according to this aspect of the present invention is usable for gene therapy.

As used herein the phrases "gene therapy" or "genetic therapy" are used interchangeably and refer to a method of therapy in which a stable or
5 transient genetic modification of a proliferative cell(s) such as a cancer cell, leads to the inhibition of proliferation of this cell.

Any one of the polynucleotides identified in Table 2 by its Gene Bank accession number can be employed according to the present invention as a polynucleotide encoding a T2-RNase. In addition,
10 polynucleotides 40 % or more homologous and/or hybridizing under mild and/or stringent hybridization conditions with the listed polynucleotides can also be employed as a polynucleotide encoding a T2-RNase, provided that the protein encoded thereby is characterized as a T2-RNase and exhibits anti-proliferative activity. Furthermore, it will be appreciated that
15 portions, mutants chimeras or alleles of such polynucleotides can also be employed as a polynucleotide encoding a T2-RNase according to the present invention, again, provided that such portions, mutants chimeras or alleles of such polynucleotides encode a T2-RNase which exhibits anti-proliferative activity.

Isolation of novel polynucleotides encoding T2-RNases is also envisaged. Such isolation can be effected using methodologies well known in the art such as, but not limited to, library screening, hybridization, PCR amplification, labeled primers, labeled degenerated primers. Both genomic and cDNA polynucleotides can thus be employed.

A polynucleotide according to the present invention can be fused, in frame, to any other protein encoding polypeptide to encode for a fused protein using methods well known in the art. For example the polypeptide can be fused to a leader sequence or a signal peptide for targeting into a 10 mammalian cell. Similarly a T2-RNase protein can be fused (conjugated) to other proteins using methods well known in the art. Many methods are known in the art to conjugate or fuse (couple) molecules of different types, including proteins. These methods can be used according to the present invention to couple a T2-RNase to other molecules such as ligands or 15 antibodies to thereby assist in targeting and binding of the T2-RNase to specific cell types. Any pair of proteins can be conjugated or fused together using any conjugation method known to one skilled in the art. The proteins can be conjugated using a 3-(2-pyridylidithio)propionic acid Nhydroxysuccinimide ester (also called N-succinimidyl 3-(2pyridylidithio)

propionate) ("SDPD") (Sigma, Cat. No. P-3415), a gluteraldehyde conjugation procedure or a carbodiimide conjugation procedure.

According to a preferred embodiment of the present invention, the polynucleotide includes one or more additional polynucleotide segments 5 harboring transcription control sequences operatively linked to the T2-RNase encoding polypeptide. Such transcription control sequences can include, but are not limited to, promoters and enhancers as further detailed hereinbelow. These transcriptional control sequences are typically operatively linked upstream to the coding region and function in regulating 10 the transcription and/or translation thereof.

According to another preferred embodiment of the present invention the polynucleotide encoding a T2-RNase is included within a eukaryotic expression vector. The phrase "expression vector" refers to a nucleic acid sequence which includes a sequence encoding a T2-RNase 15 and transcriptional control sequences and which is capable of expressing a T2-RNase within a mammalian cell.

Numerous methods for the insertion of DNA fragments into a vector, for the purposes of mammalian gene expression are known in the art and may be used to construct a T2-RNase encoding gene expression

vector including appropriate transcriptional/translational control sequences and the desired T2-RNase polynucleotide sequences. These methods may include *in vitro* DNA recombinant and synthetic techniques and *in vivo* genetic recombination. Expression of a polynucleotide encoding a T2-
5 RNase may be regulated by transcription control sequences so that a T2-
RNase is expressed in a host cell infected or transfected with the recombinant DNA molecule. For example, expression of a T2-RNase may
be controlled by any promoter/enhancer element known in the art. The promoter activation may be tissue specific or inducible by a metabolic
10 product or administered substance.

Promoters/enhancers which may be used to control T2-RNase expression within target tissues or cells include, but are not limited to, the native RB promoter, the cytomegalovirus (CMV) promoter/enhancer (Karasuyama, H., *et al.*, 1989, *J. Exp. Med.*, 169:13), the human β -actin promoter (Gunning, P., *et al.*, 1987, *Proc. Natl. Acad. Sci. USA*, 84:4831-4835), the glucocorticoid-inducible promoter present in the mouse mammary tumor virus long terminal repeat (HHTV LTR) (Klessig, D. F., *et al.*, 1984, *Mol. Cell Biol.*, 4:1354-1362), the long terminal repeat sequences of Holoney murine leukemia virus (MULV LTR) (Weiss, R., *et*

al., 1985, RNA Tumor Viruses, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York), the SV40 early region promoter (Benoist and Chambon, 1981, Nature 290:304-310), the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (RSV) (Yamamoto *et al.*, 5 1980, Cell 22:787-797), the herpes simplex virus (HSV) thymidine kinase promoter/enhancer (Wagner *et al.*, 1981, Proc. Natl. Acad. Sci. U.S.A. 78:1441-1445), the regulatory sequences of the metallothionein gene (Brinster *et al.*, 1982, Nature 296:39-42), the adenovirus promoter (Yamada *et al.*, 1985, Proc. Natl. Acad. Sci. U.S.A. 82(11):3567-71), 10 and the herpes simplex virus LAT promoter (Wolfe, J. H., *et al.*, 1992, Nature Genetics, 1:379-384).

Expression vectors compatible with mammalian host cells for use in genetic therapy of tumor or cancer cells, include, but are not limited to, plasmids, retroviral vectors, adenovirus vectors, herpes viral vectors, and 15 non-replicative avipox viruses, as disclosed, for example, by U.S. Pat. No. 5,174,993, which is incorporated herein by reference.

Several methods can be used to deliver the expression vector according to this aspect of the present invention to the target mammalian cell(s).

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For example, a suitable pharmaceutically acceptable carrier such as a physiologically appropriate solution, and which contains an effective concentration of an expression vector can be administered topically, intraocularly, parenterally, orally, intranasally, intravenously, 5 intramuscularly, subcutaneously or by any other effective means.

A physiologically appropriate solution containing an effective concentration of an expression vector can be administered systemically into the blood circulation to treat a cancer or tumor which cannot be directly reached or anatomically isolated.

10 For treating tumor masses a physiologically appropriate solution containing an effective concentration of an expression vector can be directly injected, via a needle, into a target tumor mass in amounts effective to treat the tumor cells of the target tumor mass.

Alternatively, a cancer or tumor present in a body cavity such as in 15 the eye, gastrointestinal tract, genitourinary tract (e.g., the urinary bladder), pulmonary and bronchial system and the like can receive a physiologically appropriate composition (e.g., a solution such as a saline or phosphate buffer, which is sterile except for the expression vector) containing an effective concentration of an expression vector via direct

injection with a needle or via a catheter or other delivery tube placed into the cancer or tumor afflicted hollow organ. Any effective imaging device such as X-ray, sonogram, or fiber optic visualization system may be used to locate the target tissue and guide the needle or catheter tube.

5 It will be appreciated that since a "naked" expression vector can be actively taken up by mammalian cell, uptake and targeted delivery is enhanced if the expression vector is appropriately packaged or encapsulated.

Thus, according to another preferred embodiment of the present
10 invention the pharmaceutically acceptable carrier includes a delivery vehicle suitable for the delivery of the expression vector into mammalian cells in a targeted manner.

A viral expression vector may be introduced by a delivery vehicle into a target cell in an expressible form by infection or transduction. Such
15 a delivery vehicle includes, but is not limited to, a retrovirus, an adenovirus, a herpes virus and an avipox virus. A delivery vehicle able to introduce the vector construct into a target cell and able to express T2-RNase therein in cell proliferation-inhibiting amounts can be administered by any effective method described hereinabove.

Alternatively, such a delivery vehicle can include, but is not limited to, a liposome, a micelle, an antibody or a ligand as previously described hereinabove.

- It will be appreciated that the polynucleotides herein described can
- 5 be used in the preparation of a medicament useful in inhibiting the proliferation of a mammalian cell of a mammal, by mixing the polynucleotide with an appropriate pharmaceutically acceptable carrier.

As already mentioned hereinabove, polynucleotides encoding a T2-RNase can be obtained by a variety of methods, including, but not limited

10 to, polymerase chain reaction (PCR) amplification of genomic or cDNA libraries screening using T2-RNase specific primers, using reverse transcription PCR along with T2-RNase specific primers to amplify mRNA isolated from organisms known to express T2-RNases, or directly isolating DNA sequences coding for a T2-RNase from the appropriate

15 organisms. It will be appreciated in this case that the above mentioned methods can also be used to isolate or generate any of the active forms of a T2-RNase described hereinabove.

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The purified polynucleotide can then be inserted into appropriate expression vectors or provided with the appropriate transcriptional control sequences and prepared as described hereinabove.

As further exemplified in the Examples section that follows, an assay for determining the anti-proliferative effect of a specific T2-RNase or a polynucleotide encoding same is also provided according to the present invention. Such an assay is effected by exposing proliferating cells to a T2-RNase and following their proliferative behavior over time as compared to control, untreated cells. This assay can be employed not only for selecting for the most potent T2-RNase for any specific application, but also for establishing dose response, which can be translated into initial treatment dosage in *in vivo* experiments or during treatment of a subject. It will be appreciated that this assay can also be used to determine the antiproliferative active site or portion of a T2-RNase, or to determine the activity of generated or isolated mutants which do not display ribonucleolytic activity.

Additional objects, advantages, and novel features of the present invention will become apparent to one ordinarily skilled in the art upon examination of the following examples, which are not intended to be

limiting. Additionally, each of the various embodiments and aspects of the present invention as delineated hereinabove and as claimed in the claims section below finds experimental support in the following examples.

EXAMPLES

Reference is now made to the following examples, which together with the above descriptions, illustrate the invention in a non limiting fashion.

Generally, the nomenclature used herein and the laboratory procedures in recombinant DNA technology described below are those well known and commonly employed in the art. Standard techniques are used for cloning, DNA and RNA isolation, amplification and purification. Generally enzymatic reactions involving DNA ligase, DNA polymerase, restriction endonucleases and the like are performed according to the manufacturers' specifications. These techniques and various other techniques are generally performed according to Sambrook *et al.*, Molecular Cloning - A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. (1989), which is incorporated herein by reference. Other general references are provided throughout this

document. The procedures therein are believed to be well known in the art and are provided for the convenience of the reader. All the information contained therein is incorporated herein by reference.

EXAMPLE 1

Characterization of Aspergillus niger B1 RNase and its inhibitory effect on pollen tube growth in fruit trees

5

Materials and Methods

Preparation and purification of A. niger extracellular RNase:

Aspergillus niger B1 (CMI CC 324626) was grown in liquid culture containing 1 % (w/v) wheat flour and 0.05 % (w/v) ammonium sulfate. The mixture was adjusted to pH 3.5 with hydrochloric acid and autoclaved. An inoculum of about 10^6 spores was suspended in 100 ml of medium and incubated at 30 °C in an orbital shaker, at 200 rpm for 100 hours. The growth medium was passed through a 0.2- μm membrane and dialyzed three times against 10 volumes of 2 mM sodium acetate pH 6. Two liters of dialyzed solution were loaded onto a Fractogel EMD-TMAE 650 (M) 26/10 (Merck) column, equilibrated with 20 mM sodium acetate pH 6. Bound proteins were eluted with a 500-ml linear gradient of 0-1.0 M sodium chloride in the same buffer, using a fast protein liquid chromatography (FPLC) system (Pharmacia) with a flow rate of 5 ml·min⁻¹. The fractions exhibiting the highest RNase activity were pooled and

dialyzed against 2 mM sodium acetate pH 6, and a 50-ml aliquot was loaded onto a MONO-Q 5/5 HR (Pharmacia) column, equilibrated with 20 mM sodium acetate pH 6. The elution was performed as with the EMD-TMAE column, except that only 10 ml of a 0-1.0 M salt gradient were used, at a flow rate of $1 \text{ ml} \cdot \text{min}^{-1}$.

Proteins were monitored at 280 nm and measured according to Bradford (Bradford, M.M. 1976. *Anal. Biochem.* 72:248-245), using bovine serum albumin (BSA) as a standard. Different fractions were analyzed by a 12.5 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmeli, U.K. 1970. *Nature* 227: 680-685). RNase activity was determined as previously described (Roiz and Shoseyov 1995, *Int. J. Plant Sci.* 156:37-41).

The purified RNase B1 was enzymatically deglycosylated according to the procedure described by Broothaerts et al. (Broothaerts, W.,P. et al. 1991. *Sex. Pant Reprod.* 4:258-266). The enzyme was mixed with 0.5 % (w/v) SDS and 5 % (w/v) β -mercaptoethanol and heated at 100 °C for 5 minutes. Once cooled, the reaction mixture was diluted 2.5-fold with a buffer containing 50 mM sodium phosphate pH 7.5, 25 mM EDTA, 1 % (w/v) Triton X-100 and 0.02 % (w/v) sodium azide. Peptide-

N-glycosidase F (PNGase F, Boehringer-Mannheim) was added to a final concentration of $20 \text{ units} \cdot \text{ml}^{-1}$ and incubation was performed overnight at 37°C . The sample was then mixed with sample application buffer, heated at 100°C for 5 minutes, and analyzed by SDS-PAGE using a 12.5 % gel.

RNase assays:

The optimal conditions for RNase activity were determined according to a procedure modified from Brown and Ho (Brown, P.H. and Ho, T.-H.D. 1986 Plant Physiol. 82:801-806), using a range of temperatures from 20-100 $^\circ\text{C}$ at 10°C increments, and a range of pH's from 2.5-7 at 0.5 pH units increments, established using 50 and 12 mM phosphate-citrate buffers. Samples of $10 \mu\text{l}$ were each added to $490 \mu\text{l}$ of ice-cold buffer, containing $4 \text{ mg} \cdot \text{ml}^{-1}$ yeast RNA (Sigma). Half of each sample was used as a blank by immediately adding a stop solution containing $50 \mu\text{l}$ of 0.75 % (w/v) uranyl sulfate in 25 % (w/v) perchloric acid. The remaining half was incubated for 10 minutes, following which a $50 \mu\text{l}$ of stop solution was added to each. Following centrifugation at 15,000 X g for 5 minutes, the supernatant was diluted 20-fold with distilled water and the absorbance was determined at 260 nm. One unit of

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RNase activity was determined as the amount of enzyme releasing soluble nucleotides at a rate of one A.U_{260 nm} per min.

RNase B1 was visualized by an activity gel, as previously described (Roiz and Shoseyov, 1995, Int. J. Plant Sci. 156:37-41). An SDS gel containing RNase B1 was renatured by washing twice for 15 minutes each with 20 mM acetate buffer at pH 3.5 containing 25 % (v/v) iso-propanol and then twice for 15 minutes each with buffer alone. The renatured gel containing the RNase B-1 was laid over a plate containing 0.1 % RNA and 0.8 % agarose in 20 mM acetate buffer and incubated at 37 °C for 30 minutes. The gel was then removed and the agarose plate was stained with 0.02 % (w/v) toluidine blue in water to visualize RNase activity

The effect of RNase B1 on pollen tube growth:

Peach cv. Almog pollen was germinated *in vitro* in liquid culture, as previously described (Roiz and Shoseyov, 1995, Int. J. Plant Sci. 156:37-41). Pollen grains were suspended in aliquots containing 100 µl of 15 % (w/v) sucrose, 100 µg·ml⁻¹ boric acid, 200 µg·ml⁻¹ magnesium sulfate, 200 µg·ml⁻¹ calcium nitrate and different concentrations of RNase B1. Following incubation overnight at 25 °C in a dark chamber,

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germination percentage was recorded. Pollen tube length was examined with an eyepiece micrometer.

The effect of RNase B1 treatment on pollen tube growth was also tested *in vivo*. Intact flowers of peach and in tangerine (*Citrus reticulata*, Blanco cv. Murcott) were sprayed at the early stages of anthesis with 100 units·ml⁻¹ RNase B1 in 20 mM citrate buffer at pH 3.5. In each species additional flowers at the same stage, on different branches, were sprayed with buffer alone or remained untreated as controls. Following exposure to open pollination for 48 hours, the styles were fixed in a 3:1 acetic acid to ethanol (by volume) for 24 hours, washed with distilled water and imbibed overnight in 8 M sodium hydroxide. Following thorough washing in distilled water, the styles were cut longitudinally, immersed each in a drop of 0. 1% (w/v) aniline blue in 0.1 M potassium phosphate on a slide and carefully squashed with a cover glass. Pollen tubes were observed by epifluorescence microscopy (Olympus BX40 equipped with WIB cube).

The effect of RNase B1 on fruit set:

Field experiments were done in nectarine (*Prunus persica* var. Nectarina Fantasia). Branches of 30-40 cm long, bearing approximately 10 % open flowers, were sprayed with different concentrations of RNase

B1 in 20 mM citrate buffer pH 3.5 and 0.025 % triton-X 100. Untreated branches, and branches sprayed with only buffer and triton-X 100, served as controls. The branches were sprayed at 2- to 3-days intervals during the blooming period (14 days). A month later, the number of fruit per branch was examined. For viability test, seeds were cut longitudinally through the embryo and immersed in 1 % 2,3,5-Triphenyl tetrazolium chloride in water for 4 hours at 20 °C in a dark room. Red staining indicated viable tissues.

Experimental Results

Purification and characterization of RNase B1:

A. niger grown in liquid culture produced considerable amounts of extracellular RNase B1. A temperature of 60 °C and a pH of 3.5 were found optimal for RNase activity, and were adopted as the standard conditions for subsequent RNase assays.

RNase B1 purification included three steps (Table 3). In a first step a crude filtrate contained 1000 units·ml⁻¹ and 0.05 mg·ml⁻¹ protein was obtained. The crude filtrate was passed through an EMD-TMAE column and the pooled active fractions (Figure 1, graph A) contained 0.1 mg·ml⁻¹ protein, with an RNase activity of 40,000 units·ml⁻¹. In the final step, the

pooled fractions were passed through a MONO-Q column and the active RNase fraction was eluted (Figure 1, graph B). This fraction contained a protein concentration of $1.05 \text{ } \mu\text{g}\cdot\text{ml}^{-1}$ and RNase activity of 543,000 units $\cdot\text{ml}^{-1}$. Two major protein bands, of 40 and 32 kDa, were observed following SDS-PAGE of the purified RNase B1 fraction (Figure 2). An RNase activity gel showed active bands corresponding to the 32 and the 40 kDa proteins. When subjected to PNGase F, a single protein band appeared at 29 kDa. RNase activity was retained after PNGase digestion (not shown).

TABLE 3

Purification step	Total units	Protein concentration (mg/ml)	Recovery (%)	Specific activity (units/mg protein)
Crude filtrate	2,000,000	0.05	100	20,000
EMD-TMAE column	1,120,000	0.1	56	400,000
MONO-Q column	652,200	1.05	32.6	517,143

The effect of RNase B1 on pollen tubes and fruit set:

In *in vitro*, experiments 75 % of the control pollen grains germinated and the pollen tubes reached about 0.5 mm in length. Addition of RNase B1 to the growth medium reduced the percentage of germination and the length of the pollen tubes, in a dose responsive manner (Figure 3). RNase B1 had a pronounced inhibitory effect, 50 units $\cdot\text{ml}^{-1}$, representing

0.1 $\mu\text{g}\cdot\text{ml}^{-1}$ protein, were lethal, whereas 125 $\mu\text{g}\cdot\text{ml}^{-1}$ of BSA reduced only half of pollen germinability and tube growth.

In vivo, control pollen tubes of peach were observed growing through the stigmatic tissue directed into the style 48 hours after pollination (Figure 4a). A similar effect was observed in styles treated with buffer only. In contrast, pollen grains germinated on stigmas treated with RNase B1 produced short pollen tubes, which appeared to lack any growth orientation, and failed to penetrate the stylar tissue (Figure 4b). In tangerine only a small portion of the stigmatic tissue, the diameter of which was 2-3 mm, was captured by the view field of the microscope. Therefore, only few pollen tubes were observed, as shown in Figure 5. However, the difference between the normal growth of the control pollen tubes (Figure 5a) and the irregular growth of the RNase-treated pollen tubes (Figure 5b), was clearly evident.

In nectarine cv. Fantasia, RNase B1 caused a reduction in fruit set (Table 4). In branches that remained untreated or sprayed with buffer with triton X-100, fruit set was 48.3 % and 36.3 %, respectively. It seemed that the low pH-buffer had some inhibitory effect on fruit set, however branches treated with 500 and 1000 units $\cdot\text{ml}^{-1}$ of RNase B1 set 23.3 % and

18.4 % fruits, respectively, indicating a significant thinning effect of the RNase, in a dose dependent manner.

TABLE 4

Treatment	Flowers (total number)	Fruit set (%)
Control untreated	169	48.3 a*
Control buffer	143	36.3 ab
500 units/ml RNase B1	148	23.3 bc
1000 units/ml RNase B1	106	18.4 c

* values not sharing a common letter are significantly different at P = 0.05.

In RNase B1 treated branches many undeveloped fruitlets were observed.

Viability tests showed that in the control flowers (either untreated or sprayed with buffer only), embryo tissues were stained red, (Figure 6a), whereas the tissues of embryos developed in RNase-treated flowers, stained brown indicative of necrosis (Figure 6b).

Aspergillus niger B1 extracellular RNase (RNase B1) was purified to homogeneity. It was found to contain two isoforms of 32- and 40- kDa glycoproteins, sharing a 29-kDa protein core. The optimal RNase activity was observed at a temperature of 60 °C and a pH of 3.5. In peach (*Prunus persica* cv. Almog) and tangerine (*Citrus reticulata*, Blanco cv. Murcott) the enzyme inhibited pollen germination and tube growth *in vitro* as well as *in vivo*. In field experiments, the RNase caused a reduction in nectarine

(*Prunus persica* var. *nectarina Fantasia*) fruit set and inhibited normal embryo development.

EXAMPLE 2

Inhibition of pollen germination and tube growth by T2-RNase is mediated through interaction with actin

5

The inhibition of pollen germination and tube growth by RNase is well recognized, yet the mechanism by which this enzyme interferes with the elongation process is still unclear. As such, this study set out to decipher the role of RNase B1 in interfering with the elongation process of pollen tubes.

Materials and Experimental Methods

The effect of RNase B1 on pollen tubes growth:

Anthers of lily (*Lilium grandiflorum* L. cv. Osnat) were let to dehisce for 24 hours at room temperature and than either used fresh or stored at -20 °C. RNase B1 was produced and purified from *Aspergillus niger* growth medium filtrate as described in Example 1. Pollen was germinated *in vitro* in aqueous cultures of 100 µl each, containing 7 % sucrose, 1.27 mM CaNO₃, 0.16 mM H₃BO₃, 1 mM K₂NO₃ and 3 mM KH₂PO₄ in water (Yokota and Shimmen 1994). Some cultures were supplemented with RNase B1 having 100 units/ml of RNase activity to a

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final protein concentration of 16 $\mu\text{g}/\text{ml}$. Additional cultures were supplemented with RNase that was previously boiled for 30 minutes which produced the loss of 50 % of activity, or with autoclaved RNase lacking any catalytic activity. Following 2 hours of incubation at 25 °C in the dark, pollen tube length was measured under the microscope eyepiece micrometer. The pollen tubes were stained with IKI (0.3 % I₂ and 1.5 % KI in water) to detect starch bodies.

Actively extending 1-hour pollen tubes were transferred to glass cells on the microscope stage. The pollen tubes growth pattern and organelle movement were video recorded as modified from Heslop-Harrison and Heslop-Harrison (Heslop-Harrison, J. and Heslop-Harrison, Y. 1990. Sex Plant Reprod. 3:187-194), using Applitec MSV-800 video presenter. Images were captured at 0.8 frames/sec for 8 seconds by Scion LG-3 frame grabber and then digitized and integrated by NIH *image* software. The photographs were processed using Adobe Photoshop (Adobe Systems Inc., Mountain View, CA) and Power-Point (Microsoft Co.) softwares.

The effect of RNase on pollen tube actin filaments:

Pollen was germinated *in vitro* in aqueous cultures with or without RNase. Following incubation overnight, the pollen tubes were gently

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pelleted and the growth medium was replaced with 10^{-6} M tetramethylrhodamine B isothiocyanate (TRITC)-labeled phalloidin (Sigma) in PBST buffer (150 mM NaCl, 3 mM KCl, 10 mM Na_2HPO_4 , 2 mM KH_2PO_4 and 0.02 % Tween-20). For *in vivo* observations, lily cv. Stargazer flowers were emasculated at the onset of anthesis and 0.5 ml of a growth medium containing 100 units/ml RNase were injected through the stigma into the stylar canal. Flowers into which growth medium without RNase was injected were used as a control. The liquids were absorbed into the stylar tissue for 5 hours at 25 °C, following which the stigmas were hand-pollinated by lily cv. Osnat pollen. Following 48 hours of incubation at 25 °C, each pistil was cut longitudinally and the pollen tubes were carefully excised and removed into TRITC-TBST solution and incubated for 1 hour. The incision at the stigma did not affect the pollen tubes, since their vital protoplasts were located at the distal part, protected by callose plugs. In both the *in vitro* and *in vivo* experiments the stained pollen tubes were rinsed in TBS (TBST lacking Tween-20), placed on a glass slide and observed with an epifluorescent light microscope (Olympus BX40 equipped with USH-102D mercury lamp).

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Binding of actin to RNase B1:

The interaction between RNase B1 and actin was quantified as modified from Simm (Simm, F.C. *et al.*, 1987. Eur. J. Biochem. 166:49-54). Rabbit muscle globu(G-) actin (Sigma Co.) was polymerized to filamentous (F-) actin in Buffer F (10 mM Tris pH 8, 0.1 mM ATP, 0.2 mM CaCl₂, 0.1 M KCL and 2 mM MgCl₂) for 30 minutes at room temperature. Samples of 50 µl containing each 30 µM F-actin were incubated overnight at 4 °C with 1-33 µM RNase B1. As a control, each concentration of RNase was incubated with buffer F alone. The samples were centrifuged at 15,000 g for 40 minutes and RNase activity of the supernatant was determined (Roiz, L., Goren, R. and Shoeeyov, O. 1995, Physiol. Plant. 94:585-590.).

Immunogold silver staining of RNase B1 on pollen tubes:

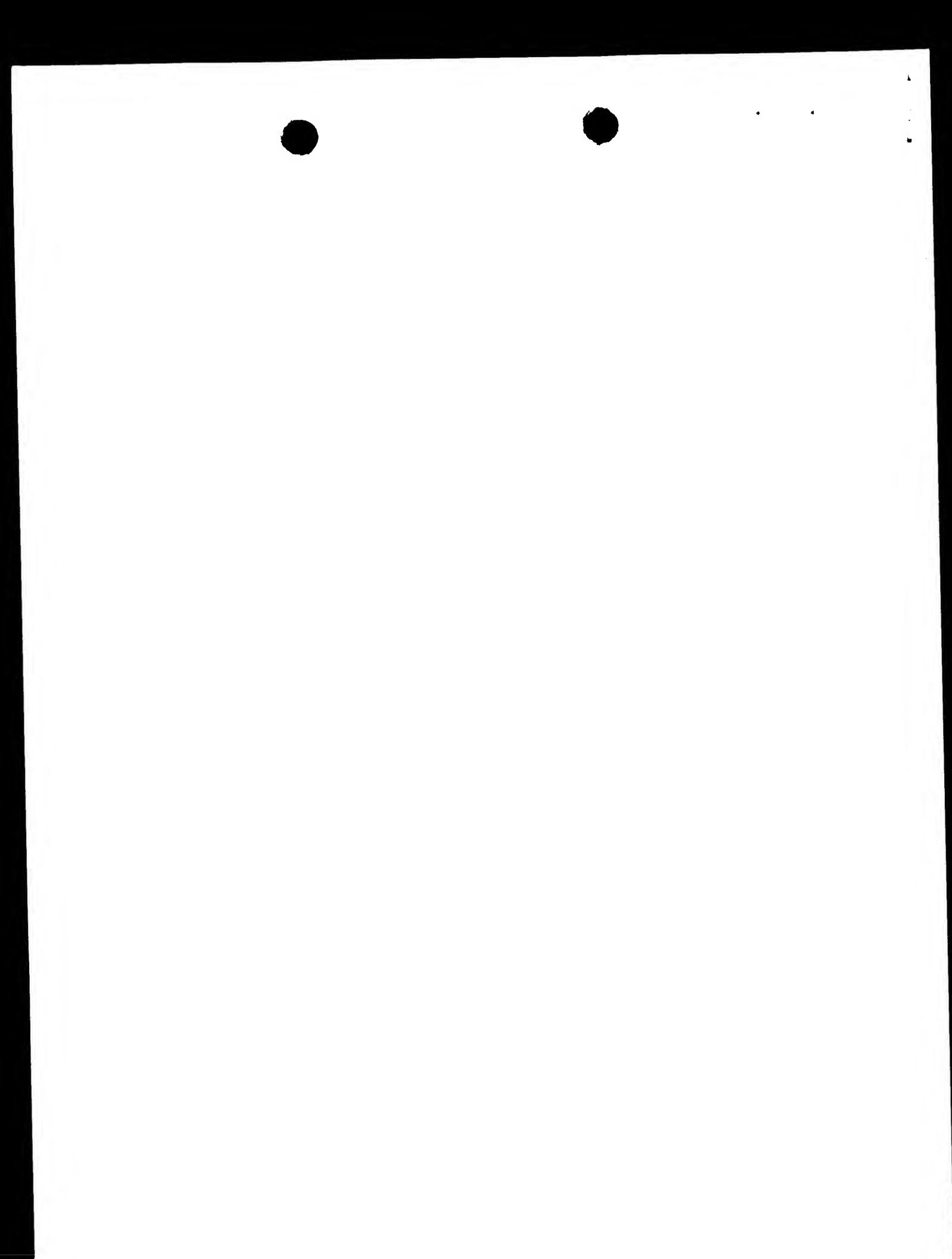
Immunogold silver stain (IGSS) was used to detect RNase attachment to lily pollen tubes. Polyclonal antibodies were raised against RNase B1 in rabbit (Aminolab). *In vitro* 2-hours lily pollen tubes were fixed overnight in 2.5 % gluteraldehyde in PBST at 4 °C. The pollen tubes were washed for 1 hour in PBST, blocked for 1 hour in PBST containing 1 % BSA and 2 % skim-milk and incubated for 1 hour in anti-RNase B1,

diluted 1:500 in PBST. Rabbit pre-immune serum (PIS) was used as control. The pollen tubes were washed three times, 10 minutes each, in PBST and then incubated for 1 hour in goat anti-rabbit IgG conjugated with 5-nm gold particles, diluted 1:100 in PBST. Following two 10 minutes-washes in PBST and one 10 minutes-wash in water, a silver-stain kit (BioCell Research Laboratories) was used for the final development of the reaction. The pollen tubes were soaked in the combined kit solutions for 10-15 minutes, washed in excess distilled water and observed under a light microscope (Olympus BX40)

Experimental Results

A control sample of lily pollen tubes, germinated *in vitro* in a growth medium without RNase, reached about 300 μm in length (Figure 7). Cultures that were treated with RNase under the same conditions, reached only 160 μm in length. Boiling or autoclaving the RNase yielded pollen tubes of 130 and 170 μm in length, respectively. The differences between the three RNase-treated groups of pollen tubes were deemed insignificant.

Starch-staining showed that amyloplasts of the control sample were observed spreading along the pollen tube, except at the tip zone



(Figure 8a). On the other hand, in RNase-treated pollen tubes IKI-stained bodies accumulated at the tip zone (Figure 8b).

The integrated video images of actively extending pollen tubes displayed the cytoplasmic flow lines (Figures 9a and 9b). In the control sample a continuous longitudinal movement was most common, as was acropetal flow at the tube periphery and basipetal flow at the center, forming an "inverse fountain" pattern beneath the tip zone (Figure 9a). The tip zone itself was occupied by much smaller bodies, mainly P-particles, the movement pattern of which was hardly observed. In RNase-inhibited pollen tubes the tip appeared swollen, with well-visible starch and lipid particles reaching the tip zone (Figure 9b). No continuous movement could be detected, but instead extended irregular images indicated cytoplasmic bodies rotating randomly.

The effect of RNase on the actin filaments distribution was examined in 1-hour *in vitro* and 48-hours *in vivo* pollen tubes. The *in vivo* pollen tubes reached about 3-4 cm long, and their TRITC-phalloidin staining was more intensive than in the *in vitro* tubes. However, The mode of the RNase effect was similar in both experiments. In the control, actin microfilaments were assembled longitudinally along the tube axis,

forming a fine network in the tip zone (Figure 10a). On the other hand, in RNase-treated pollen tubes masses of actin were accumulated at the tip cell wall (Figure 10b).

The interaction between RNase B1 and actin was quantified using Scatchard analysis. In the actin-RNase B1 experiment a regression line, intersecting with the abscissa at 0.45 (Figure 11) indicated that the RNase:actin molar ratio was 0.45, implying that two actin molecules bind to each RNase molecule.

Pollen germinated in the presence of RNase B1 were prepared for light microscopy and the location of RNase was determined by IGSS, using anti-RNase antibodies (Figures 12a-c). In pollen tubes grown without RNase (Figure 12a) or with RNase but treated with PIS (Figure 12b), the cell wall external surface was devoid of silver staining. On the other hand, in pollen tube treated with RNase B1, a clear immunogold silver stain appeared, accumulating upon the tip zone (Figure 12c).

In this study Lily (*Lilium grandiflorum*) pollen germination and tube elongation were specifically inhibited by *A. niger* RNase B1. Boiled or autoclaved RNase, lacking most of the original catalytic activity, showed a similar inhibitory effect. The results demonstrate that *A. niger*

RNase is an actin-binding protein having an inhibitory effect on pollen tube elongation. This binding which is unrelated to the catalytic activity of RNase B1 deforms the pollen tube actin filaments arrangement to thereby disrupt cytoplasmic streaming.

EXAMPLE 3

Cytotoxic effect of RNase B1 on human colon cancer cells

Since the actin binding activity uncovered for RNase B-1 in pollen tubes hinted at a possible cytotoxic activity it was decided to examine the cytotoxic effect of RNase B1 on human colon cancer cells.

Materials and Experimental Methods and Results

Cell culture:

All the experiments were performed *in vitro*. Human colon adenocarcinoma (HT29) cells were grown in DMEM medium (Biological Industries, Bet Haemek), supplemented with 10 % fetal calf serum, 1 % glutamine and 10 % Antibiotic-Antimicotic solution (Biolab). The cells were incubated at 37 °C in a humidified atmosphere containing 5 % CO₂. RNase B1 solutions were made in PBS buffer, pH 6.8.

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Preliminary cell viability assay:

Cells were incubated in 50-ml flasks. Each flask contained 2×10^5 cells in 7 ml medium, in the absence or presence of different concentrations (10^{-8} - 10^{-6} M) of RNase B1. The cells were grown for 48 hours or 72 hours, and then viable and non-viable cells were differentially counted using trypan blue staining.

In all treatments the total number of cells grown for 72 hours ($55-60 \times 10^5$) exceeded by about twofold the number of cells obtained after 48 hours culture ($25-30 \times 10^5$) (Figure 13a). The presence of RNase B1 in the growth medium did not have a significant effect on cell growth. However, a small but significance effect of the RNase B1 on the number of dead cells was found at both 48 hours and 72 hours of incubation (Figure 13b).

Clonogenicity assay I:

The long-term survival of tumor cells is characterized by their ability to divide and produce clones. Cells were preincubated for 48 hours in growth medium containing 10^{-6} M RNase B1, then trypsinized, washed and resuspended in growth medium lacking RNase B1. Prior to plating into 96-well microtiter plates, the cells were diluted to serial 5-fold

dilutions ranging between 50-to- 10^5 cells in each well (200 ml). The plates were incubated for 14 days in the conditions described above, without adding fresh growth medium following which the colonies were fixed and stained with methylene blue. The clonogenic cells in each well were numbered following clones visualization. Control cells were treated as above, but preincubated during the first 48 hours in medium lacking RNase B1.

In both treatments a similar number of colonies was observed in wells in which 100 cells were plated (Figure 14). The cytotoxic effect of the RNase B1 appeared in wells containing higher densities of cells. In wells plated with 500 cells each, the control and the RNase B1-treated cells produced 180 and 100 colonies per well, respectively. Furthermore, in wells plated with 1000 cells each the RNase B1-treated cells formed about 250 colonies per well, whereas the control cells formed numerous colonies that fused to a continuous layer, thus could not be counted and illustrated in Figure 14. Cells plated at higher densities did not survive the culture without changing media.

Clonogenicity assay II:

The ability of the tumor cells to proliferate and to colonize was examined in short vs. continuous exposure to RNase B1. The experiment was performed as described in Clonogenicity assay I using (i) control cells, (ii) cells preincubated in medium containing 10^{-6} M RNase B1 and then allowed to colonize in RNase B1-free growth medium, and (iii) cells preincubated as in (ii) and then incubated during the colonization assay in growth medium containing 10^{-6} M RNase B1. In these experiments the initial densities ranged between 250-1000 cells per well and the colonization period was 7 days.

The shorter period of incubation used in this experiment (7 days) compared to a 14 day preincubation resulted in non defused colonies, which could be distinguished even in wells containing high densities of cells. In all densities, 48 hours of preincubation in RNase B1 led to a reduction of 20-30 % in the ability of the cells to colonize, compared to the control (Figure 15). However in each density a continuous exposure to RNase B1 led to a dramatic reduction of 90 % in clonogenicity. Figures 16a-c Show that the continuous RNase B1-treated cells (Figure 16c) were smaller and less stainable than the cells that were preincubated for 48

hours in RNase B1 (Figure 16b) or the control cells (Figure 16a). This result indicate that RNase B1 affected the colonies growth rate.

Thus as is clearly shown from the results presented herein *A. niger* RNase B1 has a clear cytotoxic effect on human adenocarcinoma HT29 cancer cells. The cytotoxic effect of RNase B1 is expressed via reduction of cell clonogenicity, rather than reduction of cell viability. It is possible that the RNase B1 has a long-term effect on the tumor cells. The RNase B1 causes a reduction in the colonies growth rate compared to the control, indicating that it may affect the ability of the cells to proliferate.

Although the invention has been described in conjunction with specific embodiments thereof, it is evident that many alternatives, modifications and variations will be apparent to those skilled in the art. Accordingly, it is intended to embrace all such alternatives, modifications and variations that fall within the spirit and broad scope of the appended claims.

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WHAT IS CLAIMED IS:

1. A method of inhibiting the proliferation of a mammalian cell, the method comprising the step of administering to a treated mammal a pharmaceutical composition including, as an active ingredient, a ribonuclease of the T2 family, said composition further including a pharmaceutically acceptable carrier.
- 2.. The method of claim 1, wherein said ribonuclease of the T2 family substantially lacks ribonucleolytic activity
3. The method of claim 1, wherein the mammalian cell is a cancer cell.
4. The method of claim 1, wherein the mammalian cell is a proliferating cell associated with a proliferative disorder selected from the group consisting of papilloma, blastoglioma, Kaposi's sarcoma, melanoma, lung cancer, ovarian cancer, prostate cancer, squamous cell carcinoma, astrocytoma, head cancer, neck cancer, bladder cancer, breast cancer, lung

cancer, colorectal cancer, thyroid cancer, pancreatic cancer, gastric cancer, hepatocellular carcinoma, leukemia, lymphoma, Hodgkin's disease, Burkitt's disease, arthritis, rheumatoid arthritis, diabetic retinopathy, angiogenesis, restenosis, in-stent restenosis and vascular graft restenosis.

5. The method of claim 1, wherein said step of administering to the treated mammal said pharmaceutical composition is effected by an administration mode selected from the group consisting of oral administration, topical administration, transmucosal administration, parenteral administration, rectal administration and by inhalation.

6. The method of claim 1, wherein said ribonuclease of the T2 family is RNase B1.

7. The method of claim 1, wherein said ribonuclease of the ribonuclease T2 family is selected from the group consisting of RNase T2, RNase Rh, RNase M, RNase Trv, RNase Irp, RNase Le2, RNase Phyb, RNase LE, RNase MC, RNase CL1, RNase Bsp1, RNase RCL2, RNase Dm, RNase Oy and RNase Tp.

8. The method of claim 1, wherein said pharmaceutically acceptable carrier includes a delivery vehicle capable of delivering said ribonuclease of the T2 family to the mammalian cell of the treated mammal.

9. The method of claim 8, wherein said delivery vehicle is selected from the group consisting of liposomes, micelles and cells.

10. The method of claim 8, wherein said delivery vehicle is selected from the group consisting of an antibody and a ligand capable of specifically binding to the mammalian cell.

11. A method of inhibiting the proliferation of a mammalian cell, the method comprising the step of administering to a treated mammal a pharmaceutical composition including, as an active ingredient, a polynucleotide including a first polynucleotide segment encoding a ribonuclease of the T2 family, the composition further including a pharmaceutically acceptable carrier.

12. The method of claim 11, wherein said ribonuclease of the T2 family substantially lacks ribonucleolytic activity.

13. The method of claim 11, wherein the mammalian cell is a cancer cell.

14. The method of claim 11, wherein the mammalian cell is a proliferating cell associated with a proliferative disorder selected from the group consisting of papilloma, blastoglioma, Kaposi's sarcoma, melanoma, lung cancer, ovarian cancer, prostate cancer, squamous cell carcinoma, astrocytoma, head cancer, neck cancer, bladder cancer, breast cancer, lung cancer, colorectal cancer, thyroid cancer, pancreatic cancer, gastric cancer, hepatocellular carcinoma, leukemia, lymphoma, Hodgkin's disease, Burkitt's disease, arthritis, rheumatoid arthritis, diabetic retinopathy, angiogenesis, restenosis, in-stent restenosis and vascular graft restenosis.

15. The method of claim 11, wherein said step of administering to the treated mammal said pharmaceutical composition is effected by an administration mode selected from the group consisting of oral

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administration, topical administration, transmucosal administration, parenteral administration and by inhalation.

16. The method of claim 11, wherein said ribonuclease T2 family is RNase B1.

17. The method of claim 11, wherein said ribonuclease of the T2 family is selected from the group consisting of RNase T2, RNase Rh, RNase M, RNase Trv, RNase Ivp, RNase Le2, RNase Phyb, RNase LE, RNase MC, RNase CL1, RNase Bsp1, RNase RCL2, RNase Dm, RNase Oy and RNase Tp.

18. The method of claim 11, wherein said pharmaceutically acceptable carrier includes a delivery vehicle capable of delivering said polynucleotide to the mammalian cell of the treated mammal.

19. The method of claim 18, wherein said delivery vehicle is selected from the group consisting of liposomes, micelles and virus.

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20. The method of claim 18, wherein said delivery vehicle is selected from the group consisting of an antibody and a ligand capable of specifically binding to the mammalian cell.

21. The method of claim 11, wherein said polynucleotide includes one or more additional polynucleotide segments harboring transcription control sequences operatively linked to said first polynucleotide segment.

22. The method of claim 11, wherein said polynucleotide is a eukaryotic expression vector.

23. A pharmaceutical composition comprising, as an active ingredient, a ribonuclease of the T2 family, and a pharmaceutically acceptable carrier.

24. The pharmaceutical composition of claim 23, wherein said ribonuclease of the T2 family substantially lack ribonucleolytic activity.

25. The pharmaceutical composition of claim 23, wherein the mammalian cell is a cancer cell.

26. The pharmaceutical composition of claim 23, wherein the mammalian cell is a proliferating cell associated with a proliferative disorder selected from the group consisting of papilloma, blastoglioma, Kaposi's sarcoma, melanoma, lung cancer, ovarian cancer, prostate cancer, squamous cell carcinoma, astrocytoma, head cancer, neck cancer, bladder cancer, breast cancer, lung cancer, colorectal cancer, thyroid cancer, pancreatic cancer, gastric cancer, hepatocellular carcinoma, leukemia, lymphoma, Hodgkin's disease, Burkitt's disease, arthritis, rheumatoid arthritis, diabetic retinopathy, angiogenesis, restenosis, in-stent restenosis and vascular graft restenosis.

27. The pharmaceutical composition of claim 23, wherein said ribonuclease of the T2 family is RNase B1.

28. The pharmaceutical composition of claim 23, wherein said ribonuclease of the T2 family is selected from the group consisting of

RNase T2, RNase Rh, RNase M, RNase Trv, RNase Irp, RNase Le2, RNase Phyb, RNase LE, RNase MC, RNase CL1, RNase Bsp1, RNase RCL2, RNase Dm, RNase Oy and RNase Tp.

29. The pharmaceutical composition of claim 23, wherein said pharmaceutically acceptable carrier includes a delivery vehicle capable of delivering said ribonuclease of the T2 family to the mammalian cell of the treated mammal.

30. The pharmaceutical composition of claim 29, wherein said delivery vehicle is selected from the group consisting of liposomes, micelles and cells.

31. The pharmaceutical composition of claim 29, wherein said delivery vehicle is selected from the group consisting of an antibody and a ligand capable of specifically binding to the mammalian cell.

32. A pharmaceutical composition comprising, as an active ingredient, a polynucleotide sequence including a first polynucleotide

segment encoding a ribonuclease of the T2 family, and a pharmaceutically acceptable carrier.

33. The pharmaceutical composition of claim 32, wherein said ribonuclease of the T2 family substantially lack ribonucleolytic activity.

34. The pharmaceutical composition of claim 32, wherein the mammalian cell is a cancer cell.

35. The pharmaceutical composition of claim 32, wherein the mammalian cell is a proliferating cell associated with a proliferative disorder selected from the group consisting of papilloma, blastoglioma, Kaposi's sarcoma, melanoma, lung cancer, ovarian cancer, prostate cancer, squamous cell carcinoma, astrocytoma, head cancer, neck cancer, bladder cancer, breast cancer, lung cancer, colorectal cancer, thyroid cancer, pancreatic cancer, gastric cancer, hepatocellular carcinoma, leukemia, lymphoma, Hodgkin's disease, Burkitt's disease, arthritis, rheumatoid arthritis, diabetic retinopathy, angiogenesis, restenosis, in-stent restenosis and vascular graft restenosis.

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36. The pharmaceutical composition of claim 32, wherein said ribonuclease of the T2 family is RNase B1.

37. The pharmaceutical composition of claim 32, wherein said ribonuclease of the T2 family is selected from the group consisting of RNase T2, RNase Rh, RNase M, RNase Trv, RNase Irp, RNase Le2, RNase Phyb, RNase LE, RNase MC, RNase CL1, RNase Bsp1, RNase RCL2, RNase Dm, RNase Oy and RNase Tp.

38. The pharmaceutical composition of claim 32, wherein said pharmaceutically acceptable carrier includes a delivery vehicle capable of delivering said polynucleotide to the mammalian cell of the treated mammal.

39. The pharmaceutical composition of claim 38, wherein said delivery vehicle is selected from the group consisting of liposomes, micelles and viruses.

40. The pharmaceutical composition of claim 38, wherein said delivery vehicle is selected from the group consisting of an antibody and a ligand capable of specifically binding to the mammalian cell.

41. The pharmaceutical composition of claim 32, wherein said polynucleotide includes one or more additional polynucleotide segments harboring transcription control sequences operatively linked to said first polynucleotide segment.

42. The pharmaceutical composition of claim 32, wherein said polynucleotide is a eukaryotic expression vector.

43. The use of a ribonuclease of the T2 family in the preparation of a medicament useful in inhibiting the proliferation of a mammalian cell, the preparation comprising the step of mixing the ribonuclease of the T2 family with a pharmaceutically acceptable carrier.

44. The use of claim 43, wherein said ribonuclease of the T2 family substantially lacks ribonucleolytic activity.

45. The use of claim 43, further comprising the step of inactivating the ribonucleolytic activity of said ribonuclease of the T2 family.

46. The use of claim 45, wherein said step of inactivating the ribonucleolytic activity of said ribonuclease is effected by a method selected from the group consisting of boiling, autoclaving and chemically denaturing.

47. The use of claim 43, wherein the mammalian cell is a cancer cell.

48. The use of claim 43, wherein the mammalian cell is a proliferating cell associated with a proliferative disorder selected from the group consisting of papilloma, blastoglioma, Kaposi's sarcoma, melanoma, lung cancer, ovarian cancer, prostate cancer, squamous cell carcinoma, astrocytoma, head cancer, neck cancer, bladder cancer, breast cancer, lung cancer, colorectal cancer, thyroid cancer, pancreatic cancer, gastric cancer, hepatocellular carcinoma, leukemia, lymphoma, Hodgkin's disease,

Burkitt's disease, arthritis, rheumatoid arthritis, diabetic retinopathy, angiogenesis, restenosis, in-stent restenosis and vascular graft restenosis.

49. The use of claim 43, wherein said ribonuclease of the T2 family is RNase B1.

50. The use of claim 43, wherein said ribonuclease of the T2 family is selected from the group consisting of RNase T2, RNase Rh, RNase M, RNase Trv, RNase Irp, RNase Le2, RNase Phyb, RNase LE, RNase MC, RNase CL1, RNase Bsp1, RNase RCL2, RNase Dm, RNase Oy and RNase Tp.

51. The use of claim 43, wherein said pharmaceutically acceptable carrier includes a delivery vehicle capable of delivering said polynucleotide to the mammalian cell.

52. The use of claim 51, wherein said delivery vehicle is selected from the group consisting of liposomes, micelles and cells.

53. The use of claim 51, wherein said delivery vehicle is selected from the group consisting of an antibody and a ligand capable of specifically binding to the mammalian cell.

54. The use of a polynucleotide including a first polynucleotide segment encoding a ribonuclease of the T2 family in the preparation of a medicament useful in inhibiting the proliferation of a mammalian cell, comprising the step of mixing the polynucleotide with a pharmaceutically acceptable carrier.

55. The use of claim 54, wherein said ribonuclease of the T2 family substantially lack ribonucleolytic activity.

56. The use of claim 54, wherein the mammalian cell is a cancer cell.

57. The use of claim 54, wherein the mammalian cell is a proliferating cell associated with a proliferative disorder selected from the group consisting of papilloma, blastoglioma, Kaposi's sarcoma, melanoma,

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lung cancer, ovarian cancer, prostate cancer, squamous cell carcinoma, astrocytoma, head cancer, neck cancer, bladder cancer, breast cancer, lung cancer, colorectal cancer, thyroid cancer, pancreatic cancer, gastric cancer, hepatocellular carcinoma, leukemia, lymphoma, Hodgkin's disease, Burkitt's disease, arthritis, rheumatoid arthritis, diabetic retinopathy, angiogenesis, restenosis, in-stent restenosis and vascular graft restenosis.

58. The use of claim 54, wherein said ribonuclease of the T2 family is RNase B1.

59. The use of claim 54, wherein said ribonuclease of the T2 family is selected from the group consisting of RNase T2, RNase Rh, RNase M, RNase Trv, RNase Irp, RNase Le2, RNase Phyb, RNase LE, RNase MC, RNase CL1, RNase Bsp1, RNase RCL2, RNase Dm, RNase Oy and RNase Tp.

60. The use of claim 54, wherein said pharmaceutically acceptable carrier includes a delivery vehicle capable of delivering said polynucleotide to the mammalian cell.

61. The use of claim 60, wherein said delivery vehicle is selected from the group consisting of liposomes, micelles and viruses.

62. The use of claim 60, wherein said delivery vehicle is selected from the group consisting of an antibody and a capable of specifically binding to the mammalian cell.

63. The use of claim 54, wherein said polynucleotide includes one or more additional polynucleotide segments harboring transcription control sequences operatively linked to said first polynucleotide segment.

64. The use of claim 54, wherein said polynucleotide is a eukaryotic expression vector.

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ABSTRACT OF THE DISCLOSURE

A method of inhibiting the proliferation of a mammalian cell effected by administering to a treated mammal a pharmaceutical composition including, as an active ingredient, a ribonuclease of the T2 family, the composition further including a pharmaceutically acceptable carrier. A method of inhibiting the proliferation of a mammalian cell effected by administering to a treated mammal a pharmaceutical composition including, as an active ingredient, a polynucleotide including a first polynucleotide segment encoding a ribonuclease of the T2 family, the composition further including a pharmaceutically acceptable carrier.

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Attorney Docket: 1701/1
page 1 of 2

Combined Declaration For Patent Application and Power of Attorney

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name:

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled METHODS OF AND COMPOSITIONS FOR INHIBITING THE PROLIFERATION OF MAMMALIAN CELLS. the specification of which

(check one) is attached hereto.

was filed on _____ as Application Serial No. _____ and was amended on _____. I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the patentability of this application in accordance with Title 37, Code of Federal Regulations, § 1.56(a).

I hereby claim foreign priority benefits under Title 35, United States Code, § 119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having filing date before that of the application on which priority is claimed:

Prior Foreign Application(s)			Priority Claimed	
<u>NONE</u>			<input type="checkbox"/>	<input type="checkbox"/>
(number)	(Country)	(Day, Month, Year Filed)	Yes	No
_____	_____	_____	<input type="checkbox"/>	<input type="checkbox"/>
_____	_____	_____	<input type="checkbox"/>	<input type="checkbox"/>
_____	_____	_____	<input type="checkbox"/>	<input type="checkbox"/>

I hereby claim the benefit under Title 35, United States Code, § 120 of any United States Application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States code, § 112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, § 1.56(a) which occurred between the filing date of the prior application and the national or PCT International filing date of this application:

<u>NONE</u>	<u>(Application Serial No.)</u>	<u>(Filing Date)</u>	<u>Status</u>
_____	_____	_____	(patented, pending, abandoned)
<u>(Application Serial No.)</u>	<u>(Filing Date)</u>	<u>Status</u>	(patented, pending, abandoned)

I hereby appoint the following attorneys, with full power of substitution, association, and revocation, to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith.

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Attorney Docket: 1701/1
page 2 of 2

Continuation of Combined Declaration For Patent Application and Power of Attorney

I hereby further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that wilful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such wilful false statement may jeopardize the validity of the application of any patent issued thereon.

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Attorney Docket: 1701/1
page 2 of 2

Continuation of Combined Declaration For Patent Application and Power of Attorney

I hereby further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statement may jeopardize the validity of the application or any patent issued thereon.

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Attorney Docket: 17011
page 2 of 2

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I hereby further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statement may jeopardize the validity of the application of any patent issued thereon.

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Attorney Docket 17011
page 2 of 2

Continuation of Combined Declaration For Patent Application and Power of Attorney

I hereby further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statement may jeopardize the validity of the application of any patent issued thereon.

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SMALL BUSINESS CONCERN - NEW APPLICATION

Attorney Docket No.: 1701/1

IN THE UNITED STATES PATENT AND TRADEMARK OFFICEIn RE Application of: LEVAVA ROIZ ET AL.

Filed Concurrently Herewith

For: METHODS OF AND COMPOSITIONS FOR INHIBITING THE PROLIFERATION OF MAMMALIAN CELLSVERIFIED STATEMENT UNDER 37 CFR 1.27
CLAIMING STATUS AS A SMALL ENTITY

To The Commissioner of Patents and Trademarks:

I hereby declare that:

I am the owner of, or an official empowered to act on behalf of, the small business concern identified below:

Name of Concern: YISSUM RESEARCH DEVELOPMENT COMPANY OF THE HEBREW UNIVERSITY OF JERUSALEMAddress : 46 JABOTINSKI, 91042 JERUSALEM, ISRAEL

The small business concern identified above, together with its affiliates, employs fewer than 500 persons and qualifies as a small business concern as defined in 37 CFR 1.9(d) for purposes of paying reduced fees under 35 USC § 41(a) and § 41(b) to the Patent and Trademark Office with regard to the above-entitled invention described in the specification filed herewith.

Rights under contract or law have been conveyed to and remain with the small business concern identified above with regard to the above entitled invention.

If the rights held by the small business concern are not exclusive, each other party having rights to the invention is listed below, and no rights to the invention are held by any party who could not qualify as a small entity under 37 CFR 1.9(f), namely any person who could not be classified as an independent inventor under 37 CFR 1.9(c) if that person had made the invention, or any concern which would not qualify as a small business concern under 37 CFR 1.9(d) or a nonprofit organization under 37 CFR 1.9(e).

Full Name (Party 1) : none

Address : _____

Status : Individual Small Business Concern Nonprofit Organization

Full Name (Party 2) : _____

Address : _____

Status : Individual Small Business Concern Nonprofit Organization

I acknowledge the duty under 37 CFR 1.28(b) to file, in this application, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the issue fee due after the date on which status as a small entity is no longer appropriate.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application and any patent issuing therefrom.

X NURIT INDAR

Name of Person Signing

X N. INDAR

Signature

OF THE HEBREW UNIVERSITY OF JERUSALEM

Date

Capacity of Person Signing: SECRETARY OF THE COMPANYAddress of Person Signing: 46 JABOTINSKY ST., POB 4279, JERUSALEM 91042, ISRAEL

550E30 "TTTTSSE0

Fig 1

25

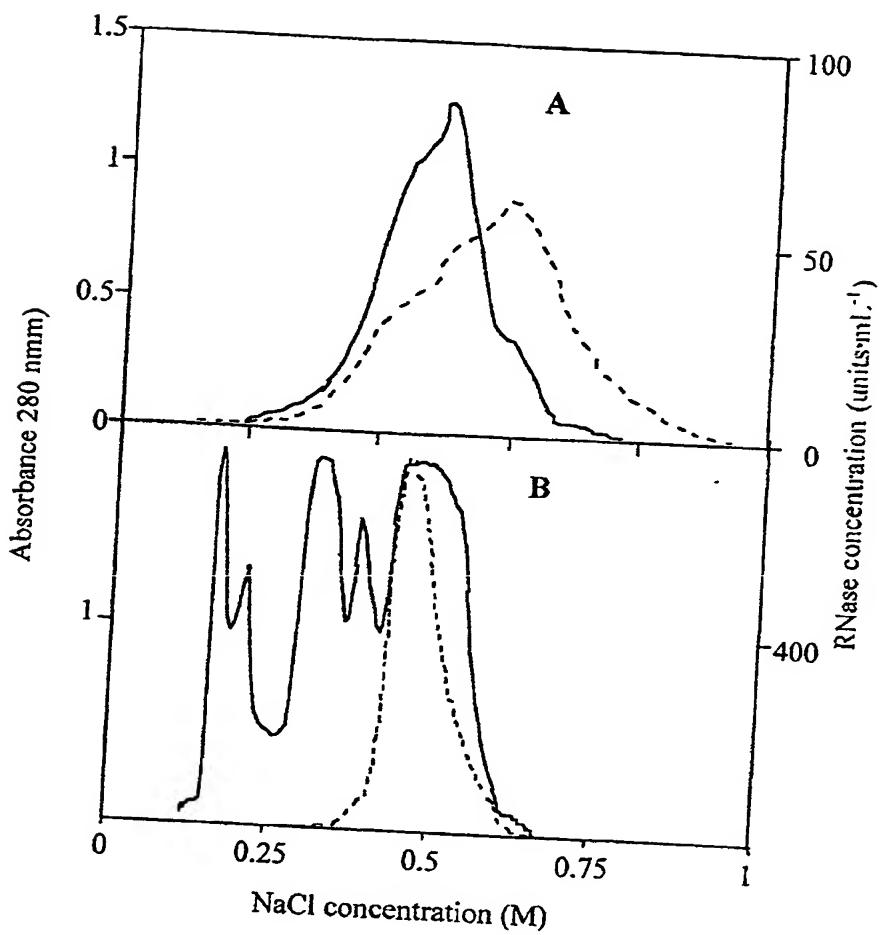


Fig 2

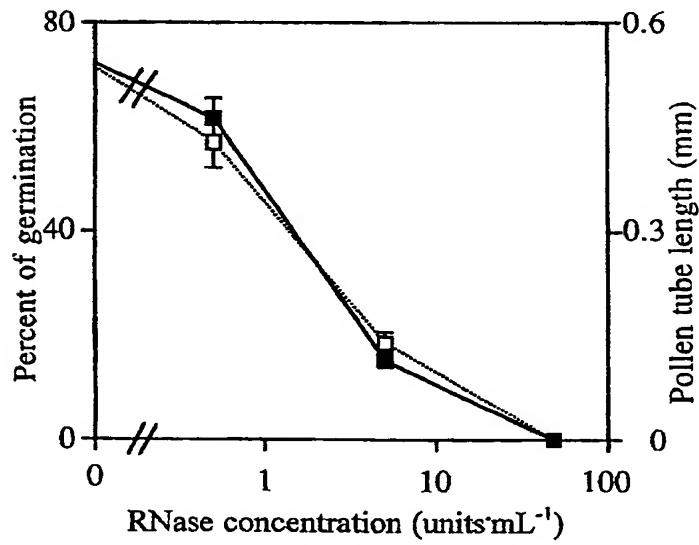
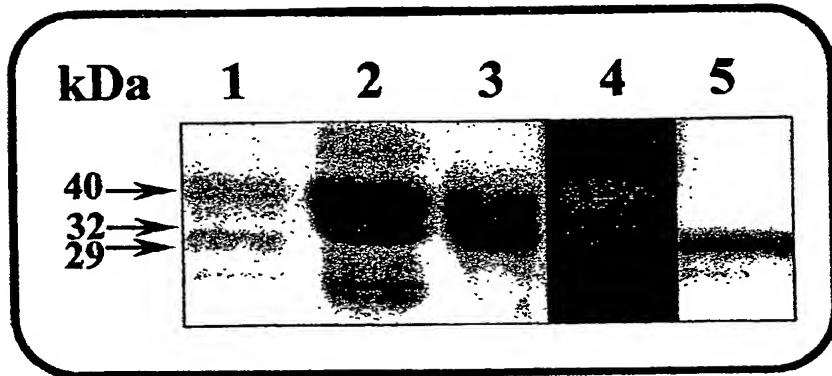


Fig 3

660E80° TTF5555555

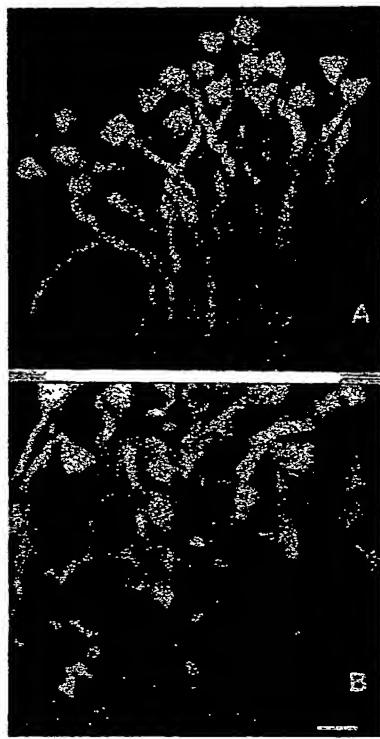
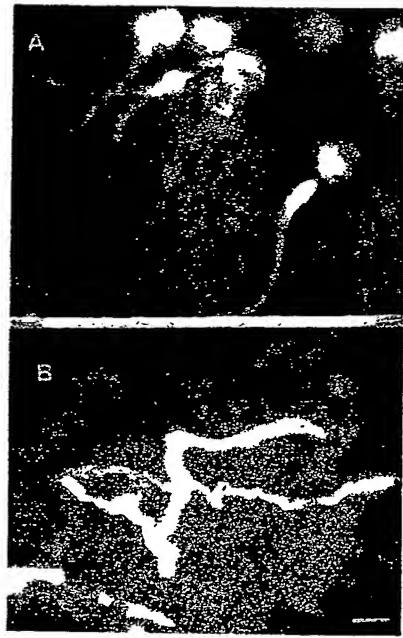


Fig 4a

Fig 4b

Fig 5a

Fig 5b



60000° T THICK

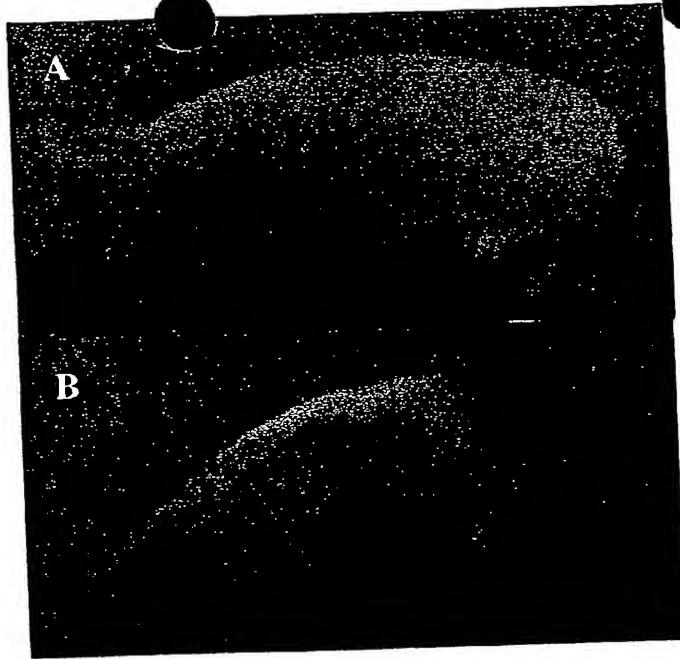


Fig 6a

Fig 6b

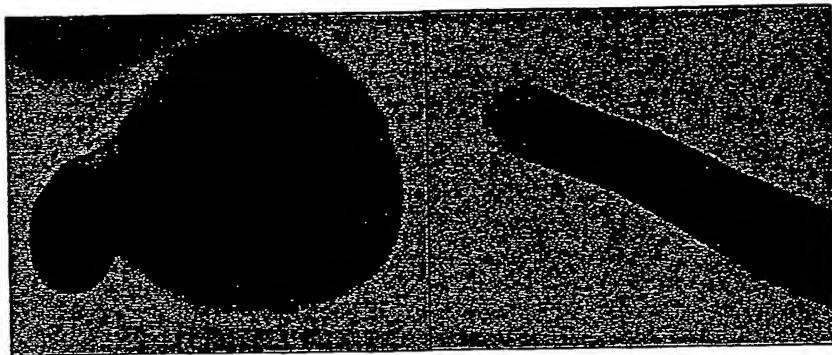


Fig 8a

Fig 8b

550550000000000000

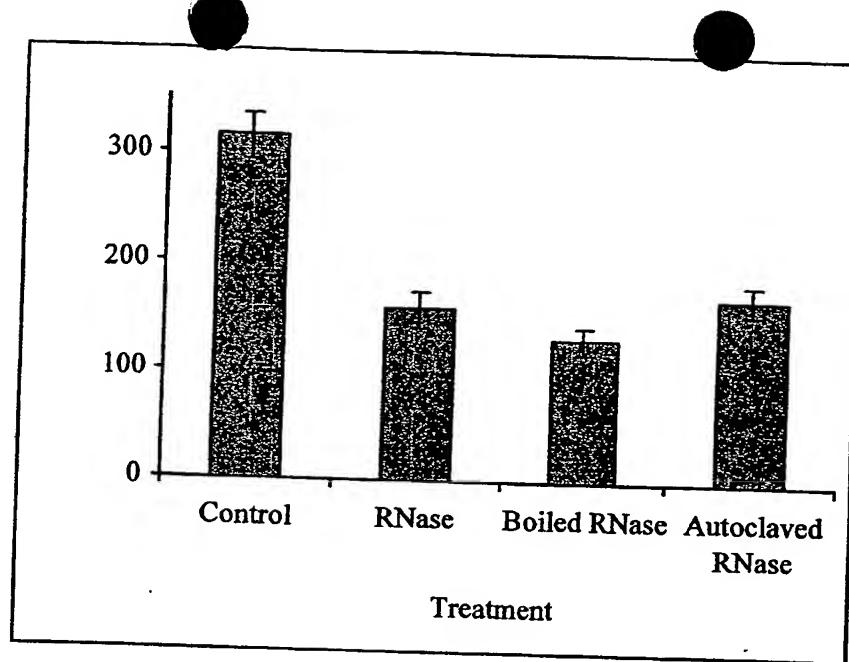


Fig 7

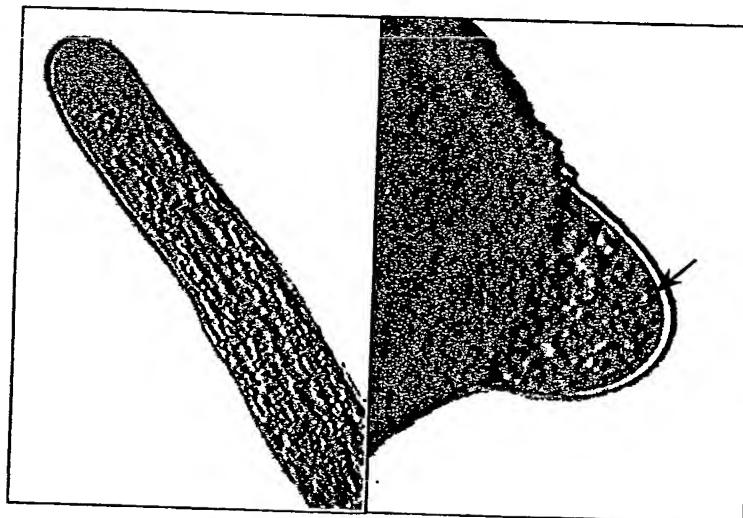


Fig 9a

Fig 9b

050550 "TT458860

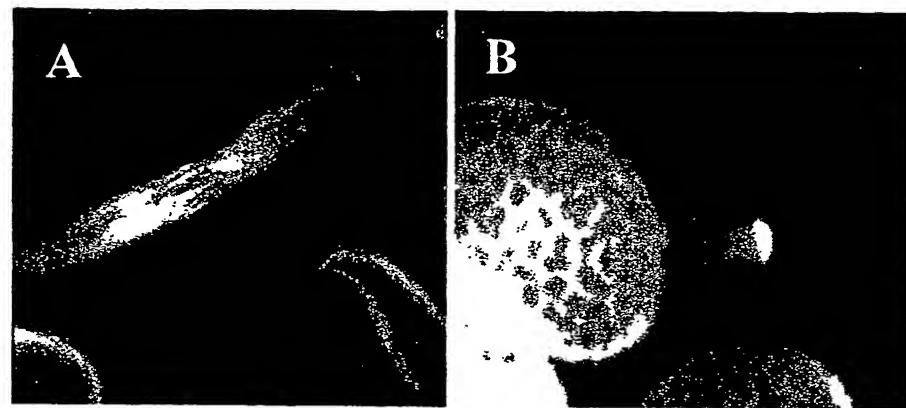


Fig 10a

Fig 10b

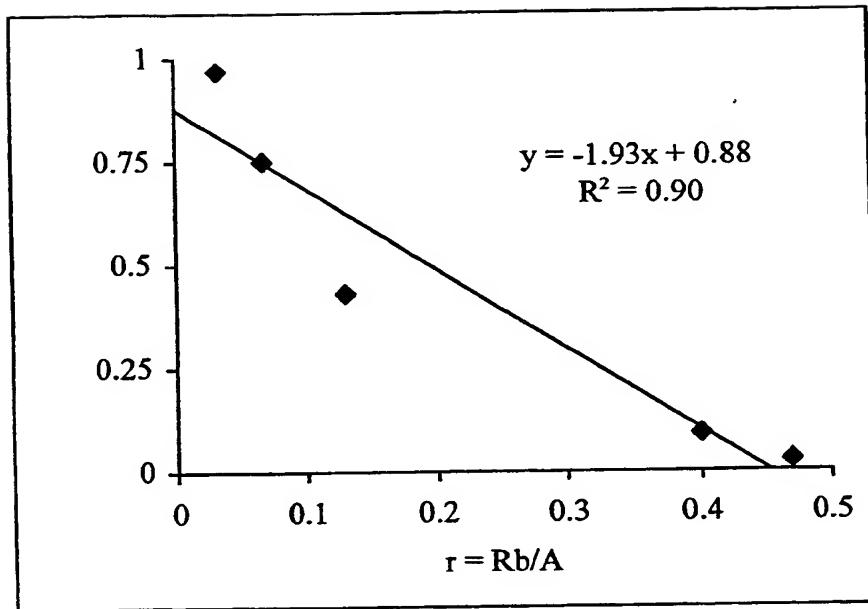


Fig 11

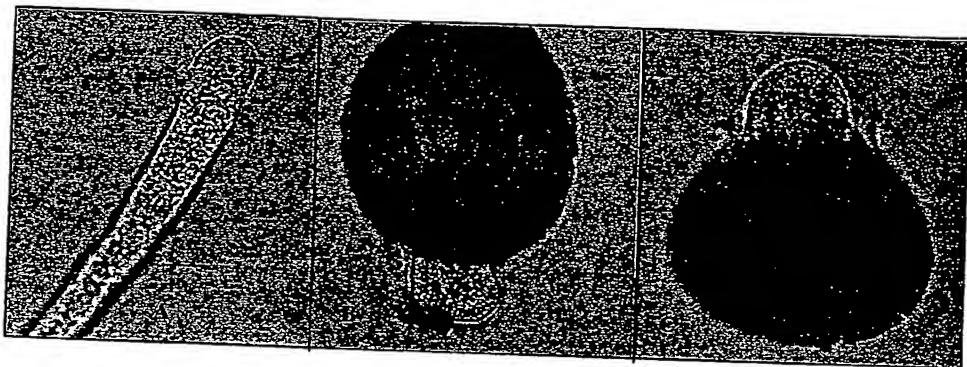
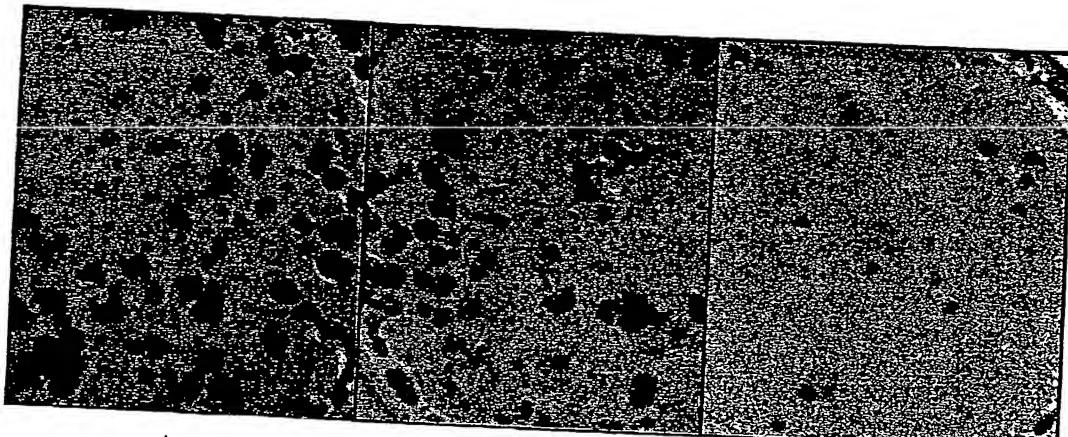


Fig 12a

Fig 12b

Fig 12c



A

B

C

Fig 16a

Fig 16b

Fig 16c

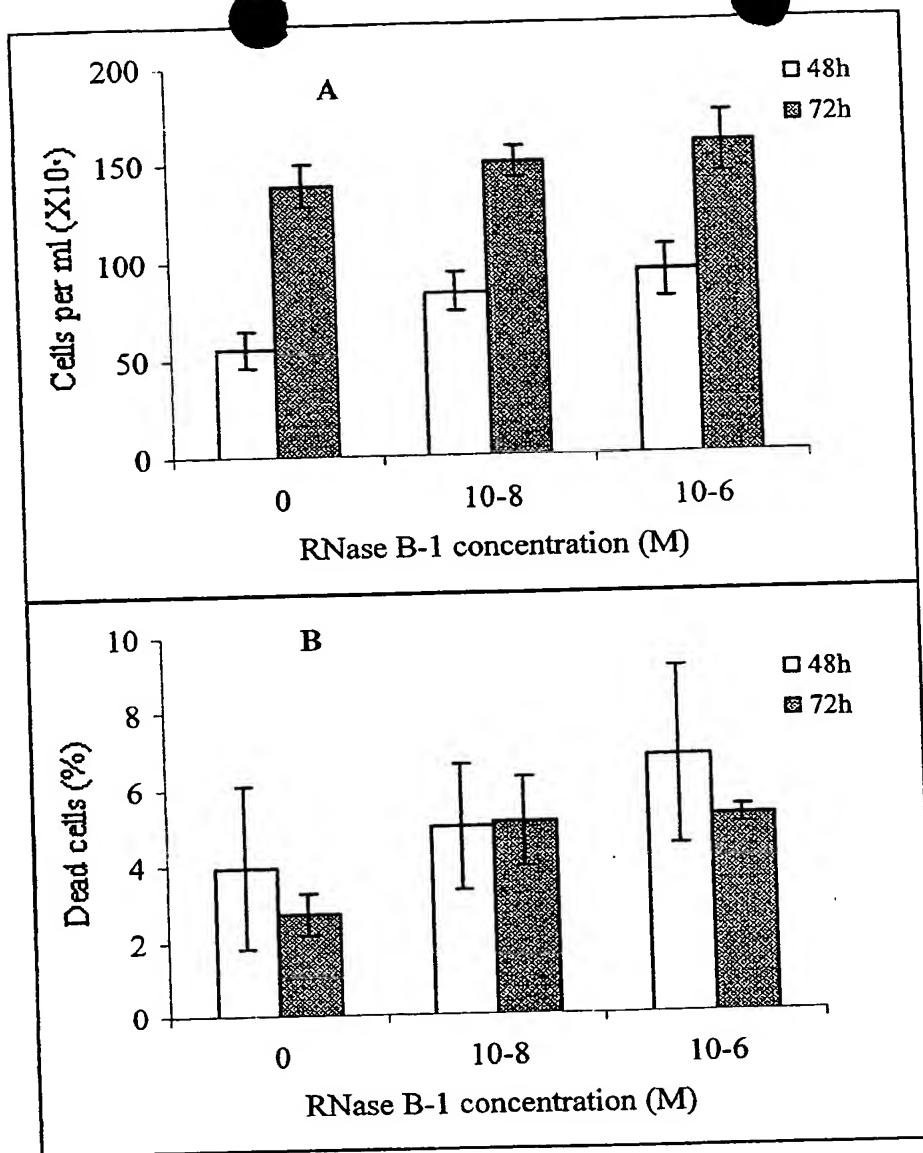


Fig 13a

Fig 13b

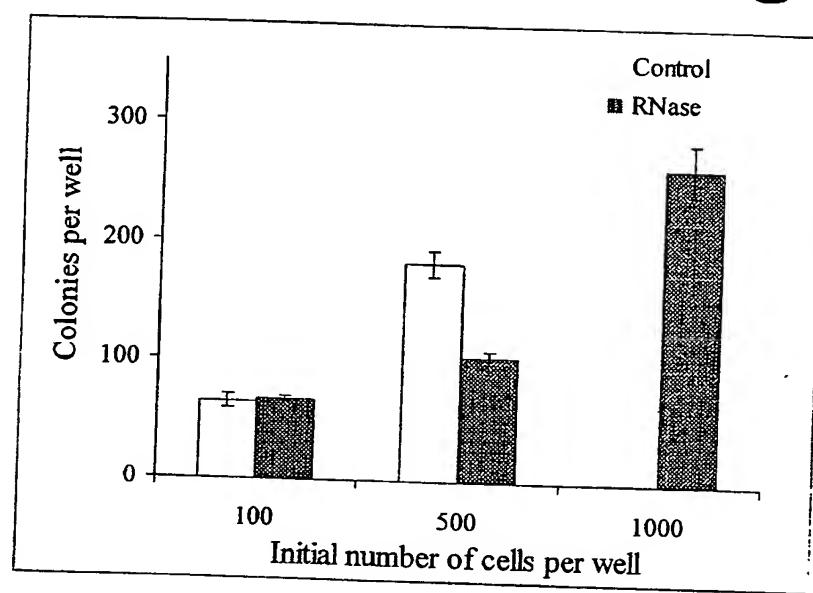


Fig 14

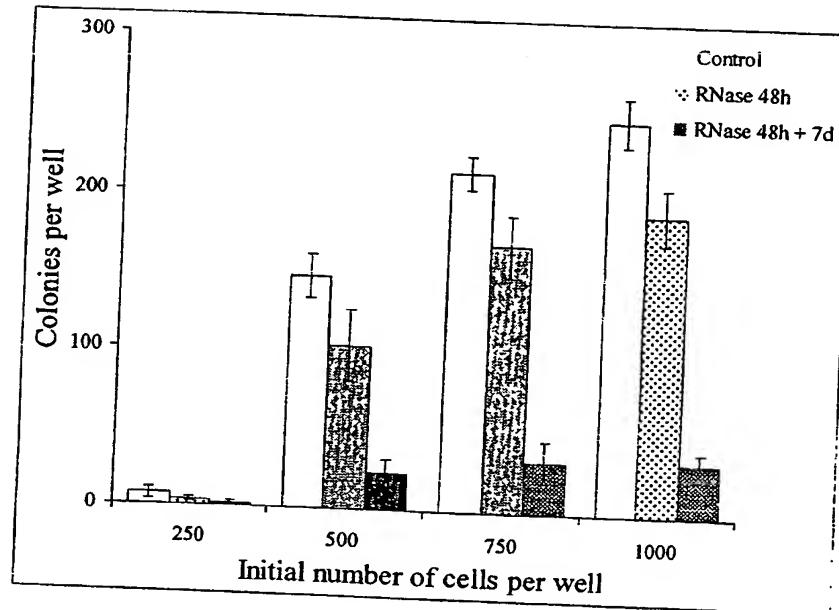


Fig 15

